5-9-2016

THE ROLE OF MAPK P38 STRESS PATHWAY-INDUCED CELLULAR TRANSLATION IN HUMAN AND MACAQUE CELLS TARGETED DURING B VIRUS INFECTION

Morgan Cook

THE ROLE OF MAPK P38 STRESS PATHWAY-INDUCED CELLULAR TRANSLATION IN HUMAN AND MACAQUE CELLS TARGETED DURING B VIRUS INFECTION

by

MORGAN COOK

Under the Direction of Julia K. Hilliard, PhD

ABSTRACT

Herpes B virus, otherwise known as *Macacine herpesvirus* 1, is a member of the family *Herpesviridae*, subfamily *Alphaherpesvirinae*, genus *Simplex*, and is closely related to human herpes simplex viruses 1 and 2 (HSV1 and HSV2). B virus is endemic in macaque monkeys, but is capable of zoonotic transmission to humans resulting in fatality in greater than 80% of untreated cases. The goal of our lab is to understand the disparity in the outcome of infection between the natural host- macaques and the foreign host- humans. An important barrier to progress is the lack of understanding of host cell: B virus interactions in response to infection. An important pathway activated by stress, known as the mitogen activated protein kinase (MAPK) p38 pathway, is activated by B virus infection. Of particular interest is its role in

regulating cellular translation via stimulation of activation of the eukaryotic initiation factor 4E (eIF4E). The activation of eIF4E is a vital rate-limiting step in translation, which can be manipulated by a variety of viruses. For example HSV1 can activate eIF4E through the p38 pathway but in the absence of this pathway eIF4E activity and viral titers are decreased. Because of the effect HSV1 has on the p38 pathway, and because B virus is a close relative of HSV1, we hypothesized that B virus also utilizes the p38 pathway to activate eIF4E in a host-dependent manner. In this dissertation, we show that the role of MAPK p38 with regard to translation is crucial to cellular processes that reduce virus replication in natural host cells, but within human cells this stress pathway appears not to play a role in reducing B virus replication. Data generated for this dissertation suggest that the p38 pathway is responsible in part for controlling the virus infection and spread within the natural host, but does not dampen virus replication in human host cells encountering the virus. Taken together, our results suggest that this pathway has at least one host-specific defense to combat B virus infection and that both cellular and viral proteins require the presence or absence of this pathway to function.

INDEX WORDS: Herpesvirus, eIF4E, Protein translation, Virus replication, ICP6, Us3

THE ROLE OF MAPK P38 STRESS PATHWAY-INDUCED CELLULAR TRANSLATION IN HUMAN AND MACAQUE CELLS TARGETED DURING B VIRUS INFECTION

by

MORGAN COOK

A Dissertation Submitted in Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

in the College of Arts and Sciences

Georgia State University

2016

THE ROLE OF MAPK P38 STRESS PATHWAY-INDUCED CELLULAR TRANSLATION IN HUMAN AND MACAQUE CELLS TARGETED DURING B VIRUS INFECTION

by

MORGAN COOK

Committee Chair: Julia Hilliard

Committee: Richard Dix

Yuan Liu

Electronic Version Approved:

Office of Graduate Studies

College of Arts and Sciences

Georgia State University

May 2016

DEDICATION

To my parents (all four of you), God knew what he was doing when he placed me in your hands. I am thankful every day to have grown up with the four of you who have never failed to keep me at my best even if that meant making sacrifices of your own. As for you Grandma, you mean more than words could possibly say. You are a huge part of why I have been successful in this endeavor and if I ever find a way to pay you back for all the things you have done, I will do so in a heartbeat. Thank you all for loving me and supporting me my whole life.

To my teacher and friend, Rachael. You know this world and my struggles perhaps better than anyone. I cannot thank you enough for instilling in me so much of your knowledge and know-how. Your advice and words of encouragement are unmatched and I hope I have made you proud just as it has made me proud to be your friend.

To my gentleman Daniel, thank you for dealing with my crazy, stressed out, emotional roller coaster. I know I have not made it easy for you and I am incredibly thankful every day that you put up with my shenanigans. Thank you so much for supporting me (and "putting a roof over my head") while I complete this degree. I love that I get to end this chapter of my life and begin a new one with you by my side. I wouldn't have it any other way. I heart you.

To my best friend Laurence, thank you for your support, advice, your shoulder to cry on, and everything along the way. There are friendships that fizzle with age and change and then there are the friendships that stand the test of time. I believe we have found the latter. I foresee us 50 years from now sitting on a front porch in our rocking chairs with our vodka sodas (that I made because yours are just vodka). We will look over at each other, smile and say, "That grass sure does look fang-like today" and no one will know what the hell we're talking about.

ACKNOWLEDGEMENTS

First and foremost, I would like to thank my mentor, Dr. Julia K. Hilliard for everything she has done for me the past five years. I came to this lab with nothing and you have given me all the tools and knowledge needed to be successful in the field of science. Know that I appreciate everything you have done more than words could possibly express.

To my committee members Drs. Dix and Liu, thank you for your unwavering support through this process. I hope I have earned your approval with the work presented in this dissertation. Thank you for asking the right questions and providing the right advice to help me focus my energy and attention on this project to make it the best it could be.

To Dr. Irina Patrusheva, thank you so much for your help with this project. Without you, this work would not have been possible. Thank you for your guidance and it has been an honor to work beside you.

To Dr. Mugdha Vasireddi, thank you for all of your wonderful help and advice. I can always count on you to be honest with me and to give me ideas that I hadn't previously thought of. You are an amazing mentor and I am thankful for your guidance and also your friendship.

To my wonderful labmates (both past and present), thank you for making the B virus lab such an enjoyable place to be everyday. You guys are truly awesome and I have enjoyed working beside you and sharing time with you. We have been through the ringer, you guys, and we have done it all together. Thank you so much for this experience and I will treasure you all. I fear I will not find such a lovable group of co-workers again in this life. You are all wonderful and I wish you all the best.

TABLE OF CONTENTS

LIST OF FIGURES

1 INTRODUCTION

1.1 B Virus

B virus (*Macacine herpesvirus* 1*)* is a member of the genus *Simplex*, the *Alphaherpesvirinae* subfamily, and the family *Herpesviridae*. Also belonging to the genus *Simplex* are human herpesviruses herpes simplex viruses 1 and 2 (HSV1 and HSV2), with which B virus shares great homology. Currently it is the only known herpesvirus endemic in nonhuman primates to be pathogenic in human hosts. The transmission of virus from animal to humans is known as zoonosis. Monkeys belonging to the genus *Macaca* are the natural hosts for this virus. Although transmission of the virus from animals to humans is rare, the mortality rate of a zoonotic infection is greater than 70% in the absence of antiviral treatments administered early in infection.

The first reported case of a zoonotic B virus infection occurred in 1932 when a physician researcher named William Brebner was bitten on the hand by a seemingly healthy rhesus macaque (*Macaca mulatta*). Young Dr. Brebner developed encephalomyelitis and succumbed to the disease approximately two weeks after the exposure event. Samples were obtained from this patient and tested on rabbits by Drs Gay, Holden, and Sabin, which resulted in similar disease progression. The virus was characterized as a herpesvirus and named B virus in memory of Dr. Brebner [1, 2].

1.1.1 Virus Structure and Life Cycle

B virus has a large, double stranded DNA genome with a high G+C content (approximately 75%), making it the simplex virus with the highest G+C content [3]. High G+C content is associated with greater DNA stability due to the presence of three hydrogen bonds between guanine and cytosine residues as opposed to the presence of just two hydrogen bonds

between adenine and thymine residues within the chain structure. Our laboratory has shown that the total genome length is approximately 156,789 bp and is organized in a manner characteristic of a simplex genome. There are six origins of DNA replication (ori regions) resulting from tandem duplication of the *oriS* and the *oriL* regions. Seventy-four genes have been identified, all of which, except one, show homology with the corresponding genes in related human herpes simplexviruses [4]. The core of the virus particle is electron dense and is surrounded by an icosapentahedral capsid with a tegument protein layer and a lipid envelope [5]. There are eleven glycoproteins attached to the lipid envelope that facilitate attachment and entry of the virus particle into the cell [4].

Like most other alphaherpesviruses, B virus infects mucosal epithelia and undergoes multiple rounds of replication in the epithelial cells. There are three classes of genes transcribed during replication that are separated by function and time of appearance post infection. The first class of genes is known as the immediate-early or alpha (α) genes, the second class consists of the early or beta (β) genes, and the last class of genes is the late/true-late or gamma/gamma^1 (γ/γ^1) genes. Infected cells eventually release new virus progeny to spread through the surrounding areas. The virus then makes its way to the sensory nerve endings where it is transferred retrograde to the nuclei in cell bodies of sensory neurons in the peripheral nervous system (PNS) [6]. Virus can also spread via syncytia formation directly from cell to cell without coming into contact with the extracellular environment, suggesting that innate cellular defenses need to exert their respective defensive reactions if the virus can escape antibodies. The virus can establish latency in the sensory neurons where it will be limited virus transcription of latency associated transcripts (LATs) and replication. Rarely does the virus travel past this site to enter the spinal cord or brain. Periodically, reactivation can occur throughout the lifetime of the host

during which time the virus will travel anterograde through the axons of sensory neurons to the dermal cells where the virions undergo replication and repeat the cycle as before. Infectious virus may then be released through the mucosa and accompanying bodily fluids [7].

In Vero cells (African green monkey kidney cells) culture, high multiplicity B virus infection shows a typical replication cycle characteristic of herpesviruses. There is an initial decrease in extracellular plaque forming units (PFUs) after addition of the virus. Intracellular virus is shown to increase six hours post infection (hpi.) and assembled virions increase in numbers through twenty-four hpi, with adjacent cells becoming infected and repeating the process over and over again whether in cell culture or *in vivo*, the latter until host defenses limit further advance. Extracellular virus progeny also increase in numbers around six hpi into each replication round, but remain relatively low (at approximately 10% of the total virus produced) for the remainder of the life cycle. Viral titers, both intra- and extracellular, begin to plateau between twenty-four and thirty-six hours hpi in cell culture. [8].

1.1.2 B virus infection in the natural host: the macaque monkey

B virus has been isolated from a variety of macaque monkeys like the stumptail, pig-tailed, Japanese, bonnet, and Taiwan macaques; however, the most commonly studied macaques are the cynomolgus and rhesus macaques (*M. facicularis* and *M. mulatta*) each used frequently in biomedical research [9]. There is an 80-100% seropositivity rate in adult macaques in captive populations that have not undergone a process to derive B virus-free offspring [10]. Macaques are usually asymptomatic, but if they are stressed or immunocompromised in any way they can exhibit herpetic lesions at mucosal sites, such as the mouth, eyes, and genitalia. Genital lesions are rarer though genital infections do occur [11, 12]. Macaques are more likely to shed virus during breeding season, immunosuppression, or primary infection [9, 10, 13].

1.1.3 Deadly B virus infection in the foreign host: the human

Zoonotic infection of humans with B virus usually occurs via direct contact with macaques or their infected bodily fluids. Infection usually occurs through bites or scratches that break the skin or by contact of the mucosa with infected bodily fluids or tissue [9, 14]. Indirect contact with a contaminated object can also result in zoonotic transmission and a human-to-human transmission event has occurred in one instance by sharing a tube of ointment that was being used to treat a bite on a spouse who was unknowingly infected with B virus [14]. Once infected there is an incubation period of anywhere from a few days to weeks, although in some cases months-to-years go by before infection becomes evident. It is widely believed that disease progression is dependent upon the site of the primary infection and the amount of virus that was introduced into the wound [9, 15, 16]. Apparent disease begins with flu-like symptoms like fever, fatigue, muscle aches, and headaches. Other symptoms that occur in some patients include lymphadenitis, nausea, vomiting, and abdominal pain. While in healthy macaques the virus stays predominantly within the PNS, in human hosts B virus eventually progresses to the central nervous system (CNS). Once the CNS has been breached, neurologic ailments begin to present such as hyperesthesias, ataxia, diplopia, agitation, and ascending flaccid paralysis depending on the region of brain or spinal cord affected [9, 14-16]. Most patients succumb to the infection once the virus reaches the CNS even with antiviral therapy and supportive care. These deaths are usually caused by respiratory failure that accompanies the ascending paralysis. The patients that survive are left with varying degrees of neurologic ailments. Latency and reactivation are also characteristics of zoonotic infection of humans, therefore patients must be vigilant in taking daily antiviral medications to prevent a reactivation, which is just as dangerous as the initial infection [17].

1.1.4 B virus as a threat both inside and outside the laboratory

Macaque monkeys are a invaluable resource in biomedical research as they are the best model for HIV infection because of the similarities between HIV and simian immunodeficiency virus (SIV) derived from sooty mangabys. Because of the use of macaques in research studies and intensive breeding practices to supply greater numbers of animals, the number of zoonotic infections of humans with B virus has increased since the initial use of macaques in research models [18]. The greatest clusters of zoonotic infection prior to the present time occurred during the production of the polio vaccine in the 1950's and 1960's. Zoonosis occurs when an endemic or natural host pathogen that controls the virus, in this case B virus in macaques, jumps as a result of close contact to invade a similar yet unfamiliar host, the human. Zoonosis implies that a human has become infected by an animal virus.. These barrier breaches usually occur between species that are closely related; however, transmission barriers can be either inherently low or artificially lowered. For example, humans coming into contact with exotic animals at zoos or parks, pets living in the same space as their owners, and more recently the development of xenotransplantation wherein animal organs are transplanted into humans [19].

The majority of viruses burdening the human population today originated in animals and include influenza viruses, human immunodeficiency virus -1 (HIV-1), dengue, and Nipah viruses [20]. Some herpesviruses have been associated with zoonotic potential such as pseudorabies virus and equid herpesvirus (EHV-1). EHV-1 has been shown to infect a variety of cells from different hosts, with human epithelial cells being among them. Zoonotic potential is an important consideration with the growing development of xenotransplantation in the medical world. One of the major barriers to progress in using xenogenic organs for transplants is the transmission of viruses from the donor animal organ to the transplant recipient [19].

There are three stages leading to successful transfer of viral disease to a new host: (I) initial single infection of new host with no subsequent infection, (II) subsequent infections that cause local chains of transmission in the new host population, and (III) epidemic or sustained endemic host-to-host transmission in the new host. During stage I, a crucial step in determining host specificity is the initial interaction between the virus and the cells encountered by the virus. Changes in entry receptors, as well as intracellular defenses, determine if the foreign host will be successfully infected [20].

Though B virus does not currently present risks in the world of xenotransplantation, it does present a clear and present danger of zoonotic transmission to the handlers and researchers that interact with macaques, to tourists visiting endemic areas, to zoo keepers and sanctuaries, and to individuals who choose to treat macaques as pets. To make matters more difficult, reactivation and shedding of B virus in macaques is difficult to detect, therefore animals that are seropositive, but asymptomatic, present the threat of zoonotic infection to individuals in close contact with the macaques. The National Institutes of Health's National Center for Research Resources initiated funding for the development of specific pathogen-free (SPF) colonies of macaques for research purposes in 1989; however, SPF status does not necessarily mean these animals are not infected since there is difficulty detecting the virus and/or antibodies against the virus in animals that are not undergoing a primary infection, or reactivating with shedding. Asymptomatic macaques also present a challenging problem [18, 21].

Based on all of this information, it is important to understand how zoonotic viruses interact differently with their foreign and natural host cells representative of those targeted at the time of virus entry, and how these interactions contribute to different host responses. Zoonotic potential is also an important factor to consider when selecting an animal model to study human

diseases. Using models like the macaque, one if not the most popular non-human primate model puts researchers and animal handlers at increased risk of contracting infectious agents, usually when in close contact with them. Using an innovative approach to the study of how zoonotic infections progress differently in their foreign and natural hosts, we developed a model of B virus infection of human and macaque fibroblasts, representative of cells encountered upon virus entry, to investigate the modulation of a signaling pathway mediated by the mitogen activated protein kinase (MAPK) p38 that results in translation of proteins [22]. The results of these experiments provides a better understanding of cellular signaling pathways that are activated as a result of virus directly or indirectly, how B virus has selected counterattacks over millennia to neutralize these cell defenses, and how humans, in the absence of co-evolving with B virus activate cellular defenses to counter virus infection. B virus is a Risk Group 4 pathogen and these types of pathogens often find the human a dead-end host, i.e., not a hospitable host that facilitates spread of the virus throughout a population, but one which most often loses the battle with the virus.

1.2 Importance of cellular signaling pathways

Cells must survey changes in their surroundings constantly and rapidly and they do this by the reception and transmission of signals that allow them to respond accordingly to the changes. There is no sensor superior to cells in this regard. Reception and transmission of signals ignite cell signaling cascades that have evolved as first line innate defenses and respond to stimuli in an autocrine and/or paracrine fashion. These cascades rely on post-translational modifications of which phosphorylation is the most common. Phosphorylation occurs via kinases, of which there are approximately 518 known to exist in human cells [23].

1.2.1 Stress Pathways: Mitogen Activated Protein Kinase (MAPK) p38

Mitogen activated protein kinases (MAPKs) are the most common kinases in signal transduction pathways and are highly conserved. There are many MAPKs responsible for a variety of cellular responses to events both intra- and extracellular in nature. The cascades begin with the MAPKKKs (MAP3Ks) that are in close proximity to membrane bound receptors and other proteins. These MAP3Ks phosphorylate a variety of MAPKKs (MAP2Ks/MKKs), depending on the type of stimulus received by the cell. The cascade ends with the terminal kinase, the MAPK. The regulation of these kinases depends on their phosphorylation and subsequent dephosphorylation of specific amino acid residues within selected proteins. There are fourteen known MAP3K isoforms and seven known MAP2K isoforms. These MAPKs can be regulated by either MAP kinase phosphatases (MKP) or dual-specificity phosphatases (DUSP). There are four sub-families belonging to the MAPKs: extracellular signal-related kinase 1/2 (ERK1/2), extracellular signal-related kinase 5 (ERK5), MAPK p38, and Jun N-terminal kinase (JNK). The MAPK p38 and JNK pathways are considered to be stress-activated protein kinases, and are therefore also notated as the SAPK pathways. They are called as such because their activation is triggered by cellular and environmental stresses, inflammation, DNA damage, and bacterial or viral infection [24]. The focus of the studies outlined in this dissertation is the MAPK p38 activated pathways. Currently, there are four known isoforms of p38: p38α (MAPK14) [25], p38β (MAPK11) [26], p38γ (SAPK (stress-activated protein kinase) 3, ERK (extracellular-signal-related kinase) 6 or MAPK12 [27], and p38δ (SAPK4 or MAPK13) [28]. These isoforms are all encoded by different genes and are expressed in different tissues, but with a degree of functional redundancy [29]. The pathway is activated by MAP3K, of which there are three: MTK1, ASK1, and TAK1. Each then phosphorylates the MAP2Ks [30]. Canonically,

MAPKs are dually phosphorylated by MAPK-kinases or MKKs. Of these, MKKs 3 and/or 6 phosphorylate the various MAPK p38 isoforms in the Thr-Gly-Tyr (threonine-glycine-tyrosine) activation loop [31, 32]. Marasa et al.discovered that MAPK p38α can also be phosphorylated by MKK4, which has been shown to increase in senescent human fibroblasts via specialized translation targeted by four microRNAs [33]. MAPK p38 phosphorylates a variety, estimated between 200-300, of proteins both *in vitro* and *in vivo* [23]. Because of the variety of proteins that can regulate p38 and that can be regulated by p38, this pathway represents a place where multiple stimulants such as cellular stress, infection, and inflammation converge and trigger different responses that reflect what is perceived as best to protect the cell or the host. Interestingly, many viruses that have long infected specific hosts have selected mechanisms by which they can thwart the host defenses at this stage of encounter.

1.2.2 MAPK p38 and downstream effectors

While other MAPKs such as extracellular-stimulated kinase (ERKs) are not associated with stress events, stressful stimuli such as bacterial or viral infection induce constantly high levels of MAPK p38 activation, unlike normal circumstances wherein p38 activation occurs at lower levels transiently [34]. The first role of MAPK p38 that was discovered was its ability to regulate pro-inflammatory cytokine synthesis in endotoxin-stimulated monocytes [35]. Kontoyiannis and colleagues demonstrated that MAPK p38α regulates protein translation in TNFα protein production. The gene encoding TNFα possesses an AU-rich element (ARE) found in the 3'-untranslated region (UTR) that when suppressed, leads to inhibition of p38α-mediated TNFα translation [36]. Among other proteins, MAPK p38 also activates several kinases involved in gene expression such as MSK1/2, MK2/3, and MNK1/2 that are in part responsible for protein translation regulation via the phosphorylation and activation of eIF4E [37, 38].

1.2.3 MAPK p38 and translation

As previously mentioned p38 phosphorylates a number of downstream effectors, which are grouped into a family of proteins called the MAPK-activated protein kinase (MAPKAPK) family. There are four subclasses of MAPKAPKs: RSK, MK, MNK, and MSK. RSKs are specifically activated by ERK and MKs are specifically activated by p38; however, MNKs (MNK1 and MNK2) and MSKs (MSK1 and MSK2) can be activated by both ERK and p38 [39]. MNK1 and MNK2 are phosphorylated on Thr197 and Thr202 residues. Studies have shown that MNK1 is inducible by growth factors, stress events, and inflammatory cytokines whereas MNK2, which is subdivided into proteins MNK2a and MNK2b is not as affected by MAPK activity. This is because MNK2a and 2b are spliced variants of the same gene wherein one possesses the MAPK binding domain (MNK2a) while the other does not (MNK2b) [39-41]. The MNKs are thought to regulate translation of cellular proteins by phosphorylating the eukaryotic initiation factor 4E (eIF4E) on serine 209 [40, 42]. The initiation factor eIF4E is known as the "cap-binding protein" and binds to the 5'-m^7GpppN cap structure on all eukaryotic messenger RNA (mRNA). This is a critical function for cap-dependent translation. Activated eIF4E participates in cellular translation as a member of the eIF4F complex along with eIF4G, which is a scaffolding protein, and eIF4A, which is an RNA helicase. Additionally, a poly adenylate-binding protein (PABP) binds the 3'-end of the mRNA as well as eIF4G to form a closed circuit with the mRNA. This is a checkpoint to ensure the mRNA is intact and functional. This complex facilitates the translation initiation by recruiting other proteins to the mRNA such as eIF3, and the 40S ribosomal subunit [43]. Regulation of eIF4E phosphorylation is, in part, managed by 4E-binding proteins or 4E-BP that binds to eIF4E and prevents it from binding to eIF4G. Gingras et al.reported that this in turn prevents eIF4E phosphorylation by sequestering it from the eIF4F

complex and ultimately MNK. They also showed that 4E-BPs can be hyperphosphorylated and inactivated by mammalian target of rapamycin (mTOR) and this allows eIF4E to bind to the eIF4F complex and subsequently interact with MNK to become phosphorylated [44]. Depending on the signaling context, either ERK or p38 may phosphorylate MNK1. When stimulated by growth factors or phorbol esters, ERK is predominantly the MAPK responsible for MNK1 phosphorylation; however, when cells are stimulated by stress events such as bacterial or viral infections or inflammatory cytokine production, p38 becomes the predominant phosphorylating kinase of MNK1 [42].

1.2.4 Viral Infection and Cellular Translation

Because viruses require materials and energy of host cells to replicate, they first target cells that are metabolically active. Viruses must recruit cellular ribosomes to translate viral mRNA into protein products needed to further their replication process, assembly, and egress. To use the cellular machinery, viruses often find ways to down-regulate cellular defenses that may thwart the infection process. They target every step of translation – initiation, elongation and termination – to take control of the host translational machinery as reviewed by Walsh and colleagues [45]. Herpes simplex virus 1 (HSV1) indirectly manipulates translation factors by preventing PABP recruitment to the eIF4F complex, which is thought to contribute to host shut-off that occurs early in infection, but restarts as infection progresses [46]. HSV1 is also capable of controlling translation by manipulating the host and viral mRNA. The DNA viruses that produce capped mRNAs with poly-adenylated tails such as poxviruses and herpesviruses have de-capping enzymes that destabilize host mRNAs upon virus entry [47]. It has long been known that HSV1 encodes a gene known as the host shut-off gene (vhs) that interacts with eIF4A and other initiation factors to increase the translation rate of both host and viral mRNAs [48] not a

primary reference but you can edit to say as described by Feng et al. Also long appreciated is that HSV1 also encodes infected cell protein 27 (ICP27), which inhibits host mRNA splicing and transport to the cytoplasm while simultaneously promoting viral mRNA export from the nucleus to the cytoplasm [49]. HSV1 also stimulates the phosphorylation of eIF4E as well as 4E-BP1 and promotes the assembly of the eIF4F complex via ICP0. When cells are treated with the p38 inhibitor SB203580, eIF4E phosphorylation via MNK1 is visibly down-regulated in western blot assays. Viral replication is also reduced in p38-inhibited cells, which suggests that phosphorylation of eIF4E by MNK1 is critical for HSV-1 protein synthesis [50]. In conclusion, HSV1, a close relative of B virus, is capable of suppressing and enhancing translation for the purpose of successful translation of viral proteins. It was logical to suspect that stress pathways in human cells may not have the advantages of those in macaque cells since macaques and B virus have co-evolved together for millennia and can co-exist peacefully, an advantage for both the virus and the host.

1.3 Summary

In summary, cells activate or inhibit a number of intracellular signaling pathways when the presence of a virus is sensed, one of which is the MAPK p38 pathway. Cells infected with alphaherpesviruses are capable of activating this cellular pathway as is evidenced by the studies done with HSV1 and its affect on p38 and downstream effectors, but little is known about the role of this pathway in the context of translation during B virus infection in the natural and foreign hosts following virus entry. This lack of knowledge presents a critical barrier to the progress of engineering antiviral interventions. Previous work in our lab determined that B virus induces specific innate immune responses when it enters the site of primary infection, viz., the epidermal and dermal layers of skin or mucosa, and these responses affect viral replication and

some lead to subsequent cytokine and chemokine production. To accomplish this, a comparative model system was developed by our laboratory using primary human foreskin fibroblasts representative of cells found at the initial site of infection in humans, and primary rhesus macaque fibroblasts, a previously unavailable, established in our laboratory through the tissue sharing program at the Yerkes National Primate Research Center. These two primary cell lines allowed for the first time the studies described in this dissertation. The MAPK pathways were targeted to study the overarching hypothesis of this dissertation because these pathways are multifunctional and are critical for cells to respond to stress, induce apoptosis for the greater good of the host, and generate inflammatory responses to recruit more extensive adaptive defenses [22]. When dermal cells become infected with HSV1 MAPKs are also activated,[51]. As previously discussed it is well known that viruses depend on host cell machinery to translate viral mRNA into protein products that enable replication and subsequent production of virus progeny. It is also established that the MAPK p38 pathway contributes to phosphorylation of eIF4E, the cap binding protein necessary for cap-dependent translation, and finally that herpesviruses are capable of regulating this pathway to benefit viral replication and protein production. The focus of this study was to investigate the role of p38 in the context of viral replication in the macaque and human at the primary infection site and how inhibiting this pathway changes viral replication and phosphorylation events of cellular translation machinery. We hypothesized that p38 regulates eIF4E phosphorylation to modulate B virus replication in macaques; particularly at the initial site of infection and that human cells lack this evolutionary advantage.

The following specific aims were designed to test this hypothesis:

Aim 1: Test the hypothesis that eIF4E phosphorylation and virus replication depends on p38 activation during B virus infection in macaques and humans

Aim 2: Test the hypothesis that interactions between B virus proteins ICP6 and Us3 and cellular translation proteins are regulated by p38 during B virus infection in macaque and human cells, representative of those found at the site of infection.

2 AIM 1: TEST THE HYPOTHESIS THAT eIF4E PHOSPHORYLATION AND VIRUS REPLICATION DEPEND ON P38 DURING B VIRUS INFECTION

Phosphorylated p38 subsequently phosphorylates MAP kinase-activated protein kinase (MNK1), which in turn phosphorylates eIF4E and our data indicate that B virus does not interfere with this stress response in either macaque or human cells. Once phosphorylated, eIF4E is able to bind to the 5'-cap of mRNA awaiting translation. After phosphorylation eIF4E becomes part of the eIF4F complex with eIF4G, a scaffolding protein, and eIF4A, an RNA helicase. This complex associates with the ribosome for translation to occur [52]. While some viruses inhibit this process, HSV-1, a close relative to B virus, drives eIF4F complex formation [50]. The experiments proposed to accomplish this aim are designed to determine if B virus regulates eIF4E phosphorylation via p38 activation and if this regulation differs between human and macaque fibroblasts.

2.1 Kinetics of MAPK p38 activation and associated protein phosphorylation for translation of mRNAs during B virus infection of cells representative of those initially infected in dermal and mucosal sites of macaques and humans.

The first experimental designs were conceived to identify at what point through 24 hours of B virus infection that p38 phosphorylation was highest, reflecting activation of cell stress pathways. Thus timecourse infections were performed at multiple independent times wherein human foreskin fibroblasts (HFF) and rhesus macaque fibroblasts (RMF) cells were infected with B virus using sufficient amounts of virus to uniformly infect the vast majority of cells used and at various times post infection, the infected cell lysates were collected for analyses. These lysates were electrophoretically fractionated using SDS-PAGE in order to transfer lysate proteins to nitrocellulose membranes to assess the relative amounts of p38 phosphorylation at different times following infection. Fibroblasts from each host were chosen as these cells make up a large portion of the cells within the dermal layer of the skin and are closest to the sensory nerve endings that ultimately become infected. In the event of a bite or scratch these cells would be among the first infected by B virus. The HFF were purchased from ATCC and, at the time there were no commercially available macaque fibroblasts, so we developed a protocol to isolate fibroblasts from necropsy samples taken from macaque monkeys. The tissues were generously provided by Yerkes Primate Research Center as part of their tissue-sharing program.

The goal of the initial experiments was not only to observe the phosphorylation and expression pattern of p38, but also the phosphorylation and expression pattern or eIF4E throughout 24 hours of B virus infection in macaque and human fibroblasts. During the first rounds of virus replication, host cells initiate innate responses that engage defenses that evolve to protect the host. Infection was done with a multiplicity of infection (MOI) 5, meaning that the

cells in the monolayer culture were exposed to approximately five plaque forming units (PFU) per ml of solution, i.e., at least five replication competent virions per cells, ensuring that 90-96% of the cells become infected at nearly the same time. This was necessary to ascertain when in the viral life cycle protein expression and phosphorylation of p38 and eIF4E would be at their highest and lowest, undiluted by cells that are not infected. The results obtained from these kinetic studies were used to determine at which time post infection the inhibition studies outlined later in this section would take place.

For the first time, as a result of these initial experiments our data revealed that phosphorylation of p38 occurs throughout B virus infection in both HFF and RMF. We discovered levels of phosphorylated p38 were significantly higher in infected HFF and RMF than in uninfected cells at the later times post infection, specifically at 18 and 24 hours post infection (**FIG 1**). Levels of phosphorylated p38 in RMF were slightly less elevated when compared to levels of the same in HFF cells, though not significantly, suggesting that B virus activates the MAPK p38 stress pathway nearly equally in both macaque and humans cells representative of the cells initially infected upon virus entry. The presence of phosphorylated p38 in uninfected cells confirmed that basal levels are needed for normal cellular activity. The increase of phosphorylated p38 late in the replication cycle suggests that the cells' response may be to control the infection by engaging stress responses, which would in turn activate several transcription factors involved in both pro- and anti-inflammatory responses. Alternatively, B virus may exploit this pathway for virus replication. To determine which scenario is taking place during B virus infection, experiments were designed to validate whether the predicted pathways were initiated following phosphorylation of p38.

The activation of the MAPK p38 pathway ultimately leads to the phosphorylation of the translational-critical eIF4E molecule, which is one of the regulators of whether capped mRNA from virus and/or cells is translated. Because eIF4E is a downstream effector of phosphorylated p38, I predicted eIF4E phosphorylation increases during B virus infection in HFF and RMF since p38 phosphorylation indeed increases between 18 and 24 hours post infection. The data from experiments designed to test this demonstrated that phosphorylation of eIF4E occurred throughout infection, but did not significantly increase during B virus infection in macaque or humans cells, when compared to levels observed in uninfected cells in spite of the increased levels of phosphorylated p38. These data may suggest that the virus can use constitutive levels of phosphorylated eIF4E to replicate, or alternatively, that the presence of eIF4E activity does not threaten B virus replication, thus there is no apparent need for the virus to have evolutionarily selected a countermeasure to block or reduce the levels of phosphorylated eIF4E (**FIG 2**).

Because phosphorylation of p38 did not significantly increase until 18-24 hours post infection, I determined that 24 hours post B virus infection in HFF and RMF would be an appropriate timepoint to sample for the next experiments. These experiments were designed to understand what would happen in each cell type to the progression of B virus infection if phosphorylation of p38 was blocked. This block would reduce or eliminate the increased activation seen late in infection and provide insight into the relevance of p38 phosphorylation and thus address whether eIF4E phosphorylation was critical to B virus replication in each cell type. To address this question the experiments described in 2.2 were designed.

2.2 Verification of p38 inhibition with SB203580 treatment of HFF and RMF during B virus infection.

Our data show for the first time that during B virus infection of macaque and human cells phosphorylation of p38 is significantly increased at 24 hours post infection. Inhibition of p38 phosphorylation at the onset of B virus infection then would alter the course of virus replication if this stress pathway serves an effective cell defense. The question posed is what happens during the initial round of virus replication if MAPK p38 stress responses cannot engage. To analyze this we added the chemical inhibitor SB203580 (600 nM) to HFF and RMF prior to, during, and after infection. Chemical inhibitors such as the pyridinyl-imidazole drug SB203580 have been successfully used to study ablation of p38 activation. This drug competitively binds to the ATP-binding pocket to block phosphorylation of p38α and p38β, but not p38γ or p38δ [53]. The inhibition via SB203580 relies on interaction with the amino acids near or inside of the ATP-binding pocket, specifically with Thr^{106} of p38α and p38β [53, 54]. In previous experiments (Figure 1), we see a basal level of p38 phosphorylation in uninfected and mock-infected cells indicating that the serum in the media is stimulating phosphorylation of p38. For this reason, prior to treatment and infection, cells were serum starved for 18-24 hours to essentially synchronize cells at the G_0-G_1 interface so that all cells would be at nearly the same place in their cell cycle at the time virus was added. This was to ensure that any observed event was triggered by the presence of virus and/or activation of p38, and not driven by the presence of serum within the medium. In uninfected and mock-infected cell lysate (MCL)-treated HFF, our data demonstrate that no p38 phosphorylation occurs. These data indicate that p38 was not activated and therefore neither the serum starvation, nor the presence of uninfected VERO cell lysate has triggered a stress response. The MCL treatment group was a necessary control because the B

virus stock used in these experiments was prepared by first infecting VERO cells, then lysing them to release virus particles. Therefore, MCL is representative of the addition VERO cell lysate along with B virus being applied to the cellular monolayer during infection, but for MCL without the presence of virus. In HFF and RMF infected with B virus, we see a marked expression of phosphorylated p38, indicating that B virus has triggered a stress response within the cells, thus there was no difference between macaque and human skin cells at this stage of infection. We observed significant reduction in phosphorylation of p38 in BV infected cells treated with SB203580 when compared with cells infected with BV in both HFF and RMF in the presence of p-p38, validating that SB203580 effectively decreased p-p38 levels (**FIG 3**). Because of the reversible properties of the inhibitor, complete reduction was difficult. Chemical inhibitors are often useful in scientific research when studying the effect of a certain protein within a system. When the activity of that protein is effectively reduced or inhibited, we can determine how the system responds to the absence of that particular protein, and or the subsequent pathways engaged, and from that we can surmise the role of a specific cellular protein during virus replication.

This experiment demonstrated that we successfully reduced and even eliminated phosphorylation of p38 in both HFF and RMF 24 hours post B virus infection. It was now possible to initiate experiments to investigate the effects of p-p38 on other factors, particularly those involved in translation of viral and cellular mRNA in HFF and RMF during B virus infection. We designed experiments to test whether p-p38 was critical for eIF4E phosphorylation, anticipating that in the absence of activating the MAPK p38 pathway, mRNA translation would be restricted to existing basal levels of p-p38. Recall that data from previous experiments demonstrated that eIF4E phosphorylation was not significantly upregulated or

down-regulated during B virus infection in either cell species. To see if p-p38 plays a role in maintaining the steady-state of eIF4E phosphorylation, we analyzed phosphorylation levels of eIF4E in the presence and absence of p-p38 during B virus infection in cells from each species, macaque and human.

2.2 Inhibition of p38 phosphorylation does not significantly alter eIF4E phosphorylation in neither HFF nor RMF during B virus infection.

Previous studies showed that HSV1 altered translation events either by altering mRNA or interaction with translation proteins [50]. Data from Walsh and Mohr demonstrated that HSV1 requires phosphorylation of eIF4E by MNK1 for replication in human fibroblasts and when these fibroblasts are treated with the p38 inhibitor SB203580 viral titers decrease, as does eIF4E phosphorylation. Their data demonstrated that p-p38 is critical for at least part of the viral replication cycle, suggesting that HSV1 exploits the stress pathway to enhance virus replication. In this case, instead of the pathway proving to be an effective cellular defense, it has been co-opted by the virus to increase virus replication. Because the process of HSV1 replication has successfully exploited a cell defense, an appropriate candidate protein to analyze is the 5'-end cap-binding protein eIF4E, which is a translation initiation factor downstream of p-p38 activity. When activated, p38 phosphorylates eIF4E kinase MNK1, which in turn phosphorylates eIF4E [23, 52]. Because eIF4E is a crucial part of the translational processes, we designed experiments to determine if B virus exploits p-p38 to modulate virus replication in the macaque host, but perhaps not in the human host via phosphorylation of eIF4E.

From these experiments our data revealed no change in phosphorylated eIF4E when the MAPK p38 pathway activation was inhibited compared with samples prepared in the presence of p38 (**FIG 4**). This is to say that there was not a significant increase nor was there a significant

decrease. If p38 phosphorylation leads to the subsequent phosphorylation of eIF4E, then inhibiting p38 activity should have decreased eIF4E phosphorylation; however, this was not observed. This data suggests that either eIF4E phosphorylation is not affected by p38 activity or that another protein, either cellular or viral in nature, is aiding in maintaining eIF4E activity in the absence of p38. Though MNK is the only known cellular protein responsible for phosphorylating eIF4E, there is another MAPK that is capable of phosphorylating MNK. Extracellular signal-related kinase (ERK) is a MAPK stimulated by non-stressful stimuli such as hormones, growth factors, etc. and also phosphorylates MNK. For this reason, we thought it necessary to test the hypothesis that ERK was phosphorylating MNK in the absence of p38.

2.3 ERK is phosphorylated at early times post B virus infection, but is not phosphorylated at late times post B virus infection in HFF and RMF.

Extracellular signal-related kinase (ERK) is a MAPK mainly activated by growth factors and phorbol esters and is thought to be a key regulator in cell proliferation, whereas p38 is more involved in the stress response and is activated by stressful stimuli such as infection, inflammatory cytokines, and environmental stress. Despite being activated by different stimuli, both MAPKs are capable of phosphorylating MNK1, which in turn phosphorylates eIF4E [55]. Because of its potential to phosphorylate MNK1, it is possible that it is also in part responsible for MNK1 activation along with p-p38; therefore, we next determined whether or not ERK is activated during a B virus infection. Analysis of our data revealed that ERK was phosphorylated in both the MCL-treated and B virus infected HFF and RMF cells, indicating that ERK activity is not downregulated in the early stages of B virus infection (0-6 hours pi). Until 6 hours post infection, we observed robust phosphorylation of ERK in both infected and uninfected cells; however, by 18 hours post infection levels of ERK phosphorylation decreased significantly in B

virus infected cells indicating that stress responses have been initiated and non-stress related pathways shutting down. By 24 hours post infection, ERK phosphorylation is almost completely gone in both cell types. At this time post infection, the cytopathic effect (CPE) of the B virus infection has increased to 4+ with syncytia formation, blebbing, and cell lysis. These data indicated that most cells were dying or undergoing apoptosis in an effort to stunt the spread of virus (**FIG 5**). These results were predicted because ERK is not a MAPK that usually participates in stress responses. These data suggests that at early times post infection, the infection is somewhat under control or has not caused sufficient damage to warrant a stress response. Alternatively, specific viral proteins may interfere with the activation of the stress responses associated with the MAPK p38. By 18h and 24h post infection, the virus has caused enough damage to induce the cellular stress responses and either directly or indirectly also decreased activity of non-stress associated pathways. Once we determined that ERK is not activated at late times post infection, we tested the hypothesis that ERK is phosphorylated during B virus infection in the absence of p38.

2.4 MAPK p38-independent phosphorylation status of ERK in B virus infected HFF and RMF at 24h p.i.

Literature suggests that MNK1 is activated only by p38 or ERK. [55]. So far we have shown that p38 phosphorylation is significantly increased during B virus infection and that ERK phosphorylation is significantly decreased during B virus infection, each observed particularly at late times post infection. In the absence of p-p38, p-ERK levels are down-regulated in human cells infected with HSV1, but it is possible that p-ERK could still phosphorylate MNK1, which in turn phosphorylates eIF4E to allow viral mRNA translation to proceed during B virus infection independently of p-p38 [56]. In our experiments, we observed that ERK

phosphorylation is apparent in untreated and MCL-treated HFF and RMF, which confirms the findings shown in Fig 5. ERK is not phosphorylated during B virus infection in the presence or absence of p-p38 activity in HFF and RMF (**FIG 6**). These data indicate that ERK phosphorylation occurs independently of p-p38 and is not responsible for maintaining eIF4E phosphorylation during B virus infection in the absence of p38. These data suggested that there is some other protein that is stabilizing eIF4E activity, raising the possibility that another cellular protein(s) or a viral protein may ensure the presence of p-eIF4E. The latter possibility will be addressed in the experiments designed to accomplish the second aim of this dissertation. Thus, our next question at this point in the designed studies was whether or not a negative regulator of eIF4E, 4E-BP1, was being regulated by p-p38 activity during B virus infection in each cell type. Because eIF4E phosphorylation does not require p-p38 activity in either cell types, we designed experiments to investigate eIF4E regulation via other cellular mechanisms. Having evaluated possible positive regulators, p38 and ERK, it was time to look at potential negative regulators.

2.5 MAPK p38 is critical for phosphorylation of 4E-BP1 in RMF, but does not affect phosphorylation of 4E-BP1 in HFF: A critical species divergence during B virus infection

Numerous investigations in multiple laboratories have shown that phosphorylation of eIF4E is inhibited by the 4E-binding proteins (4E-BP). When hypophosphorylated, 4E-BP1 binds to eIF4E, it prevents eIF4E association with eIF4G. Upon activation, the cellular protein mammalian target of rapamycin (mTOR) phosphorylates 4E-BP1, which causes the release of eIF4E, allowing the interaction between eIF4E and eIF4G. With eIF4E no longer bound to 4E-BP1, MNK1 can phosphorylate eIF4E and translation can proceed [50]. Because eIF4E phosphorylation occurred independently of p38 activity during B virus infection of macaque and

human cells, we designed experiments to evaluate whether or not 4E-BP1 phosphorylation requires MAPKp38 activation during infection.

There are seven known phosphorylation sites on 4E-BP1, five of which are conserved among all species. The simultaneous phosphorylation of sites Thr 37, Thr 46, Ser 65, and Thr 70 are crucial for separation of 4E-BP1 from eIF4E. This simultaneous phosphorylation process occurs in a specific order as well. Two sites are initially phosphorylated on 4E-BP1, which primes the protein for subsequent phosphorylation on the Thr 70 site and lastly the Ser 65 site, which results in the release of 4E-BP1 from eIF4E [57, 58]. For this reason, we chose to analyze the phosphorylation status of 4E-BP1 at residue Ser 65 as it is the last site phosphorylated for complete dissociation from eIF4E.

From the collected data, we observed that in B virus infected HFF and RMF cells, 4E-BP1 hyperphosphorylation occurred at basal levels in uninfected and MCL-treated HFF and RMF, indicating that basal levels are necessary for everyday cell function. When HFF and RMF were infected with B virus (MOI 5), levels of hyperphosphorylated of p38 increased, indicating that eIF4E becomes available for phosphorylation. This was expected, as 4E-BP1 must be inactivated to release eIF4E so that it can be phosphorylated and translation can commence, which is critical for translation of viral mRNAs essential for the production of structural proteins of the virus. When p38 activity is ablated in HFF during infection, 4E-BP1 hyperphosphorylation is maintained, indicating that this occurs in a p38-independent manner. In RMFs infected with B virus in the absence of p-p38, hyperphosphorylation levels were significantly reduced to levels observed in uninfected RMFs (**FIG 7**). These data suggest several scenarios. The reduction in hyperphosphorylation can indicate more non-phosphorylated 4E-BP1 bound to eIF4E. Because our data indicated that eIF4E phosphorylation was independent of p-p38 levels in B virus

infected RMF, this is unlikely. Alternatively, this finding could also be indicative of proteosomal degradation of 4E-BP1. After hyperphosphorylation, 4E-BP1 molecules are tagged for proteosomal degradation if the phosphate groups are not promptly removed by a phosphatase. Because of this, we designed experiments to determine if total levels of phosphorylated 4E-BP1 were being decreased in the absence of p-p38 (**FIG 8**). The results of this experiment revealed that the total level of phosphorylated 4E-BP1 remained relatively similar in all of our treatment groups strongly suggesting that phosphorylated 4E-BP1 is not being degraded by the proteasome during B virus infection of either cell type.

2.6 Viral titers increase in the absence of p38 in a host-dependent manner

Because of our findings showing the p38-independent phosphorylation status of eIF4E and p38-dependent phosphorylation of 4E-BP1 during infection in the presence and absence of p38 in HFF and RMF, we quantified virus replication. We predicted that the cellular stress pathway MAPK p38 is activated by B virus infection to down-regulate virus replication in an effort to protect the host. To determine whether or not cell proteins critical for effective mRNA translation are affected by the MAPK p38 pathway during B virus infection, we inhibited this pathway to evaluate its role in productive viral replication. Our data from this experiment revealed that in HFF, viral titers do not change in the absence of p38, suggesting that in these primary human cells stress pathways fail to modulate virus replication. In RMFs, viral titers increased by almost one log in the absence of p-p38 compared with cells in which the MAPK p38 pathway was active (**FIG 9**). These data suggest for the first time that p38 plays a regulatory role in viral replication in the natural host, but fails to modulate B virus infection in the foreign host cells at the site of virus entry. This is the first insight into potential mechanisms that have been selected in the natural host after the opportunities of millennia of co-evolution. This

observation provides valuable insight into how zoonotic viruses gain a deadly advantage over humans where there have been no evolutionary opportunities for the virus and host to adapt to each other.

2.7 Aim I Conclusions

Because viruses require cellular machinery to replicate, we selected the MAPK p38 stress pathway to study its function during zoonotic viral invasion, using a cell-based model of species-specific infection. In the absence of adequate animal models to study zoonotic infection and the costly *in vivo* studies in the natural host of B virus, we established a cell model system to investigate potential differences that may exist in the natural versus the human host cells targeted upon virus entry. The use of this cell model system allows the use of many essential controls and the isolation of specific innate defense pathways. The particular interest for this dissertation research was to enhance our understanding of cell translational machinery and thus we focused our studies on the translation factor eIF4E, which is crucial to cap-dependent translation, and additionally on 4E-BP1, one of the critical regulators of eIF4E in addition to p38 and ERK. In our findings, we showed that eIF4E phosphorylation during B virus infection of macaque and human cells occurred independently of stress pathways. Our data suggest that although B virus infection of human cells targeted at virus entry does not significantly impact eIF4E phosphorylation to enhance or decrease mRNA translation via the MAPK p38 pathway, macaque cells may effectively use this pathway to limit virus replication to levels that can be controlled by host defenses. Investigators have long appreciated that macaques quickly produce serum antibodies within 10-14 days post B virus infection, whereas humans produced only low and inconsistent levels of these antibodies. The reason for this remains unknown. We sought to explore whether innate cellular defenses may differ between macaque and human hosts,

particularly since the relationship between B virus and the macaque host have evolved over millennia, providing many opportunities for selection that allows co-existence of the virus and macaque host, whereas humans have not had this opportunity.

Because 4E-BP1 hyperphosphorylation was identified as a critical mechanism which HSV1 and human hosts selected during their co-evolution, we asked whether this particular aspect of infection was conserved between B virus and its natural host, macaques. Using our developed comparative cell culture model system, we discovered that without effective MAPK p38 stress pathway activation, RMFs infected with B virus were observed to have a significant decrease of in 4E-BP1 hyperphosphorylation levels comparable to the levels seen in uninfected cells while levels in HFF remained unaffected. This data suggested to us that in RMF there should be increased free 4E-BP1 available to bind and sequester eIF4E; however, phosphorylation of eIF4E was not significantly affected in RMF. These observations beg the question of what is preventing this free, hypophosphorylated 4E-BP1 from binding eIF4E and preventing translation. To ensure that the decrease in hyperphosphorylated 4E-BP1 was not due to proteosomal degradation, we compared the levels of total 4E-BP1 during infection in the presence or absence of p-p38 and discovered that levels of total 4E-BP1 were maintained independently of the MAPK p38 stress pathway activation. These observations suggested to us that a viral protein may dephosphorylate 4E-BP1 and or/block degradation.

In the experiments designed to better understand the dynamic of the host:virus interactions, we observed that in B virus infected RMFs, 4E-BP1 hyperphosphorylation increased compared to uninfected cells. This suggested that eIF4E was free to be phosphorylated and participate in translation. When p38 phosphorylation is blocked, we expected that eIF4E phosphorylation would decrease because it is downstream of p-p38 but that was not what we

observed. Instead eIF4E phosphorylation did not significantly change whether the MAPK p38 stress pathways were activated or not in our experiments. These data suggested to us that there is another protein responsible for eIF4E phosphorylation and this could potentially be a viral protein.

The data presented here have shown for the first time that macaque-specific MAPK p38 stress pathways affect host:virus interactions differently than human-specific MAPK p38 pathways. To our knowledge, this is one of the first reported observations of species differences in host:virus interactions involving the MAPK p38 cellular stress pathway. In primary RMF cells, which are representative of the cells at the site of primary infection in the natural host, the absence of a viable MAPK p38 response results in almost a log increase in virus titers; however, in HFF, which are representative of the cells at the site of primary infection in the human host, B virus replication is unfettered by the activation of the MAPK p38 stress pathway. This data suggests for the first time that p38 plays a key role in dampening virus replication in RMF but not in B virus infected primary HFF cells.

Several independent investigators have provided evidence that certain specific viral proteins have the ability to control translation machinery or mimic the machinery. For example, HSV1 viral proteins have been shown to manipulate formation of the eIF4F complex [29], and hantavirus provides a protein that mimics the complex to enhance viral mRNA translation [30]. Therefore it is possible that like HSV1, a B virus protein can interfere with one or both of these processes involving eIF4E. Our experiments described in this dissertation demonstrated that p-p38 does not regulate eIF4E phosphorylation in a host-dependent manner during B virus infection; however, the host-dependent regulation of 4E-BP1 during infection in the absence of p38 activity was demonstrated. These findings potentially serve to illuminate how this zoonotic

virus regulates cellular pathways differentially between hosts. These data demonstrate how a zoonotic virus, in this case B virus, can regulate a cellular process in the natural host with which it has co-evolved, but not in the foreign human host and suggest how these interactions can contribute to the host-specific differences in the outcome of virus infection. Once it is known how a zoonotic virus interacts with its natural host cell and its human host cells, we will then have the possibility of designing intelligent antiviral therapeutics and vaccines.

2.8 Figures

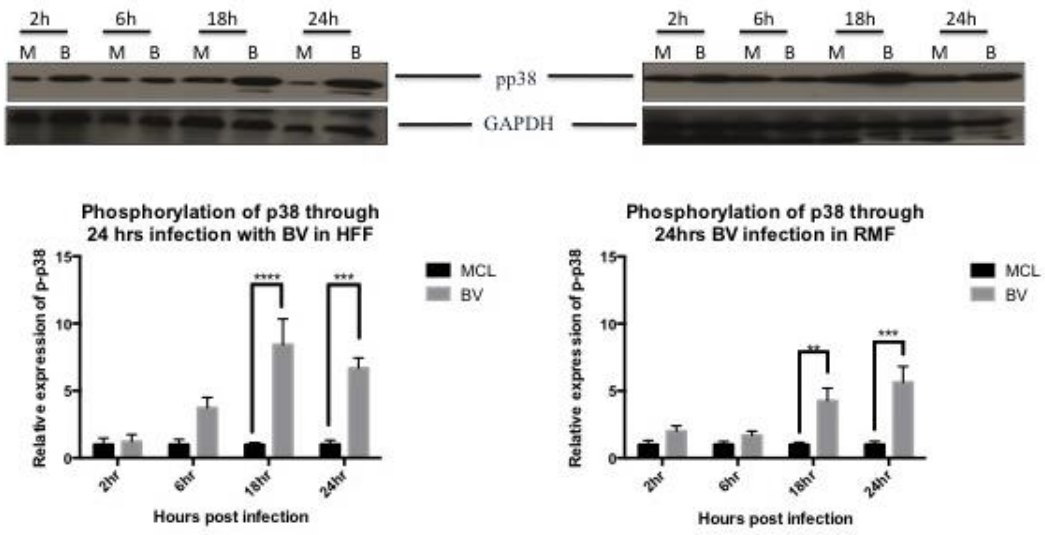

Figure 1: Phosphorylated p38 through 24 hrs B virus infection in HFF and RMF

Figure 1: Phosphorylated p38 through 24 hours post B virus infection in HFF and RMF. (Top) Western blot analysis of HFF and RMF treated with mock cell lysate (M) or infected with B virus at MOI 5 (B) at various times through 24 hours. (Bottom) Densitometric analysis of

phospho-p38 normalized to the amount of GAPDH in each sample. Figures are representative of three independent experiments in duplicate.

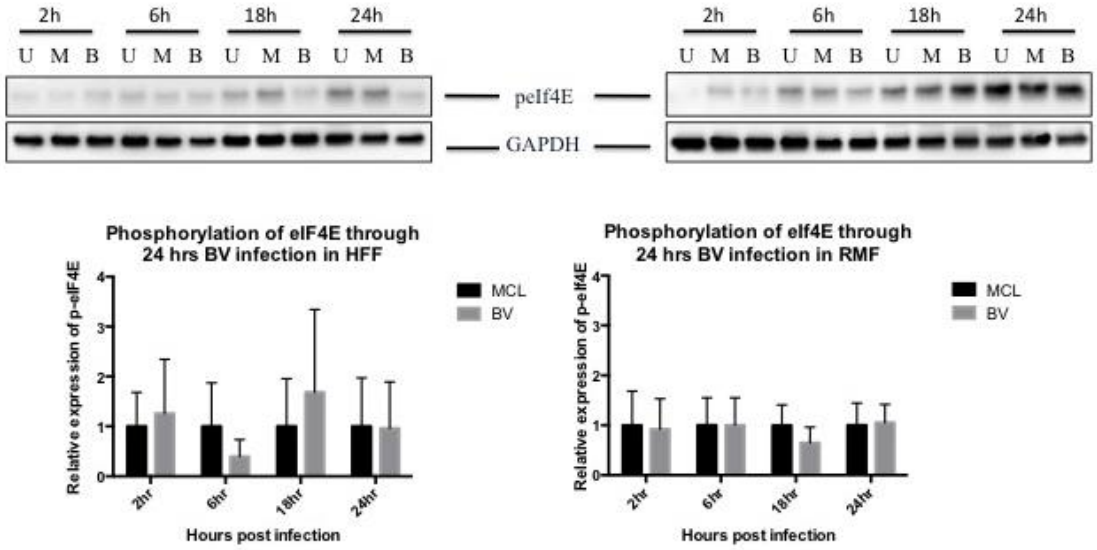

Figure 2: Phosphorylated eIF4E through 24 hrs. B virus infection in HFF and RMF

Figure 2: Phosphorylated eIF4E through 24 hours post B virus infection in HFF and RMF. (Top) Western blot analysis of HFF and RMF uninfected (U), treated with mock cell lysate (M) or infected with B virus at MOI 5 (B) at various times through 24 hours. (Bottom) Densitometric analysis of phospho-eIF4E normalized to the amount of GAPDH in each sample. Figures are representative of three independent experiments in duplicate.

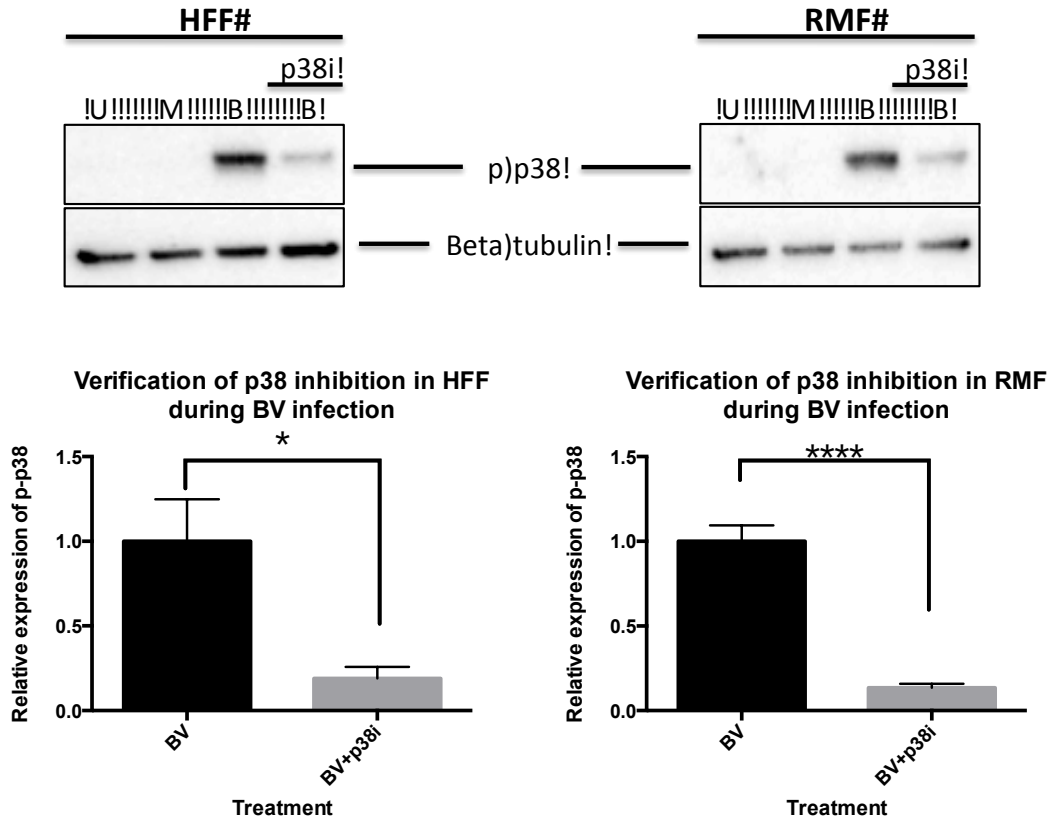

Figure 3: Verification of p38 inhibition via SB203580 in HFF and RMF

Figure 3: Verification of p38 inhibition via SB203580 in HFF and RMF. (Top) Western blot analysis of HFF and RMF uninfected (U), treated with mock cell lysate (M), infected with B virus at MOI 5 (B), or infected with BV and treated with SB203580 (p38i) for 24 hours. (Bottom) Densitometric analysis of phospho-p38 normalized to the amount of beta tubulin in each sample. Figures are representative of three independent experiments in duplicate.

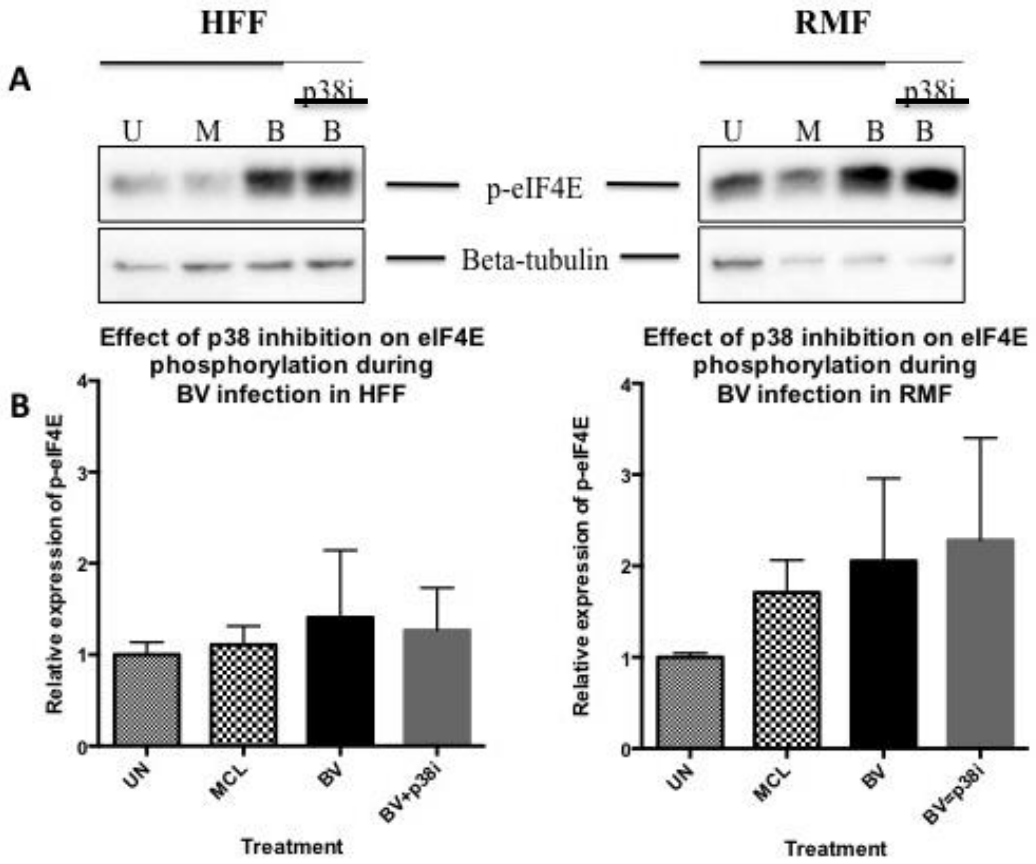

Figure 4: eIF4E phosphorylation occurs independently of p38 in HFF and RMF

Figure 4: eIF4E phosphorylation occurs independently of p38 in HFF and RMF. A) Western blot analysis of HFF and RMF, uninfected (U), treated with mock cell lysate (M), infected with B virus at MOI 5 (B), or infected with BV and treated with SB203580 (p38i) for 24 hours. B) Densitometric analysis of phospho- eIF4E normalized to the amount of beta tubulin in each sample. Figures are representative of three independent experiments in duplicate.

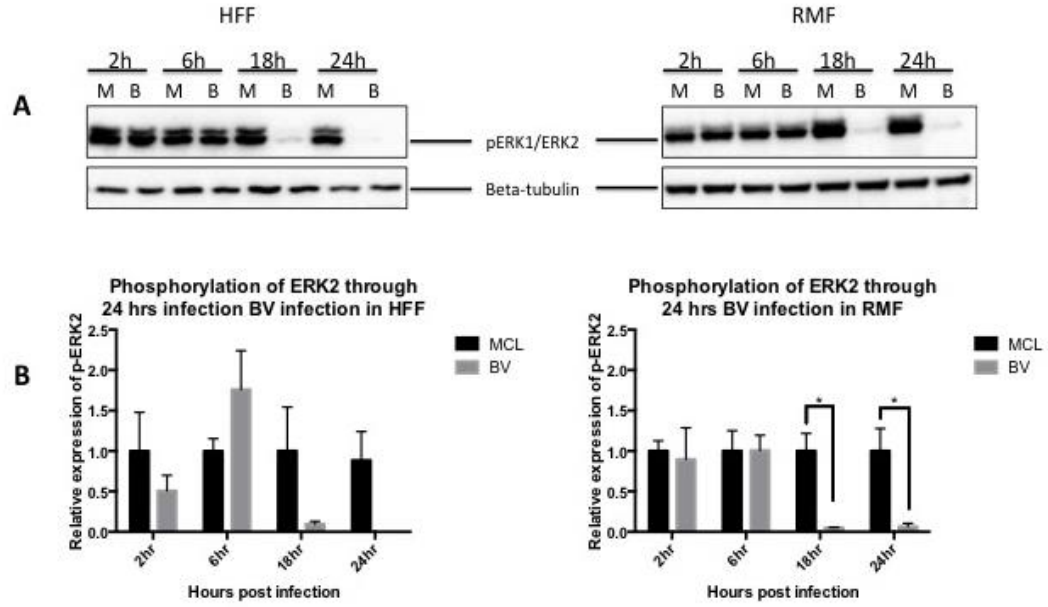

Figure 5: ERK phosphorylation is downregulated late in B virus infection.

Figure 5: ERK phosphorylation is downregulated late in B virus infection. A) Western blot analysis of HFF and RMF, treated with mock cell lysate (M), or infected with B virus at MOI 5 (B), at various time points through 24 hours. B) Densitometric analysis of phospho-ERK1/2 normalized to the amount of beta tubulin in each sample. Figures are representative of three independent experiments in duplicate.

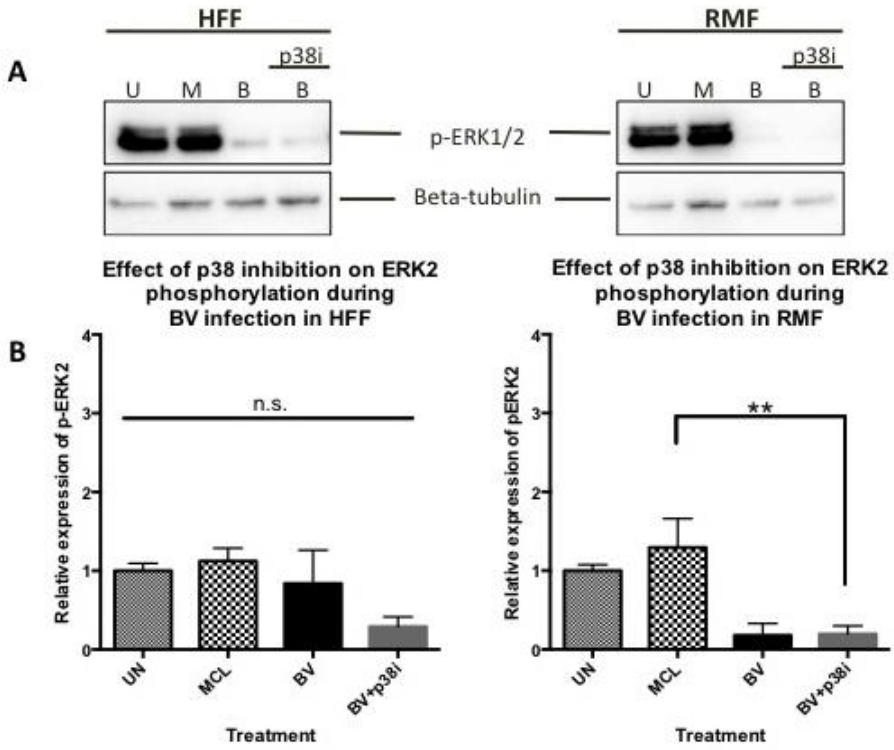

Figure 6: ERK phosphorylation is unaffected by p38 during B virus infection

Figure 6: ERK phosphorylation is unaffected by p38 during B virus infection. A) Western blot analysis of HFF and RMF, uninfected (U), treated with mock cell lysate (M), infected with B virus at MOI 5 (B), or infected with BV and treated with SB203580 (p38i) for 24 hours. B) Densitometric analysis of phospho- ERK2 normalized to the amount of beta tubulin in each sample. Figures are representative of three independent experiments in duplicate.

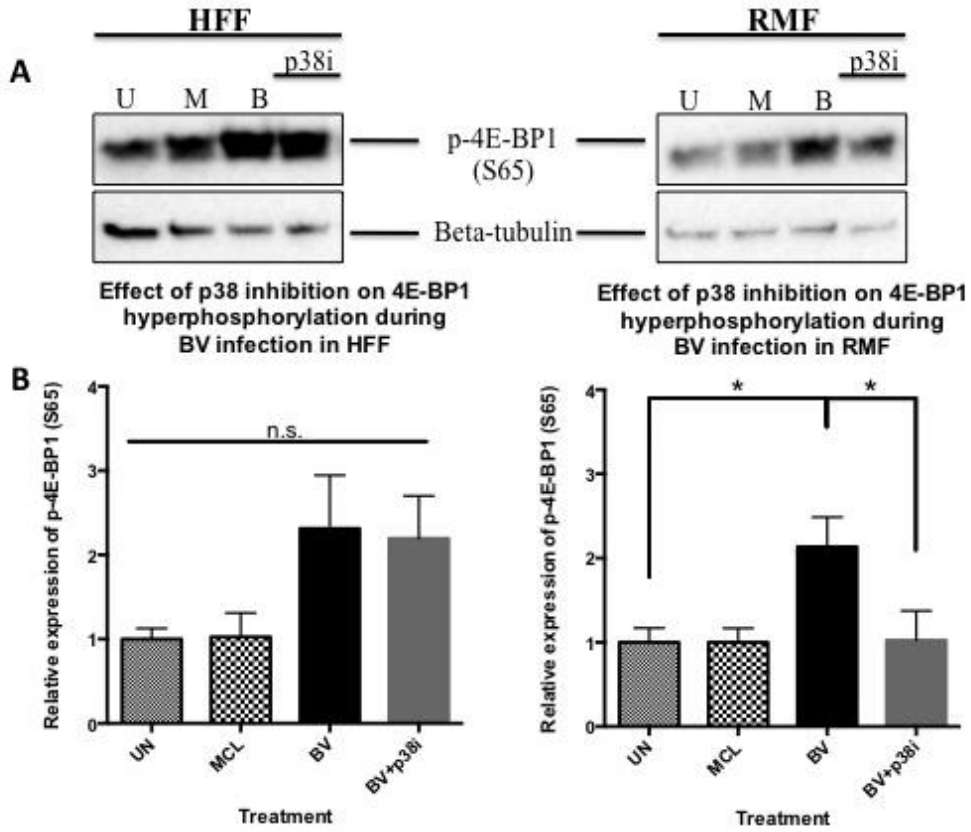

Figure 7: 4E-BP1 hyperphosphorylation requires p38 in the natural host

Figure 7. 4E-BP1 hyperphosphorylation requires p38 in the natural host during B virus infection. A) Western blot analysis of HFF and RMF, uninfected (U), treated with mock cell lysate (M), infected with B virus at MOI 5 (B), or infected with BV and treated with SB203580 (p38i) for 24 hours. B) Densitometric analysis of phospho-4E-BP1 (S65) normalized to the amount of beta tubulin in each sample. Figures are representative of three independent experiments in duplicate.

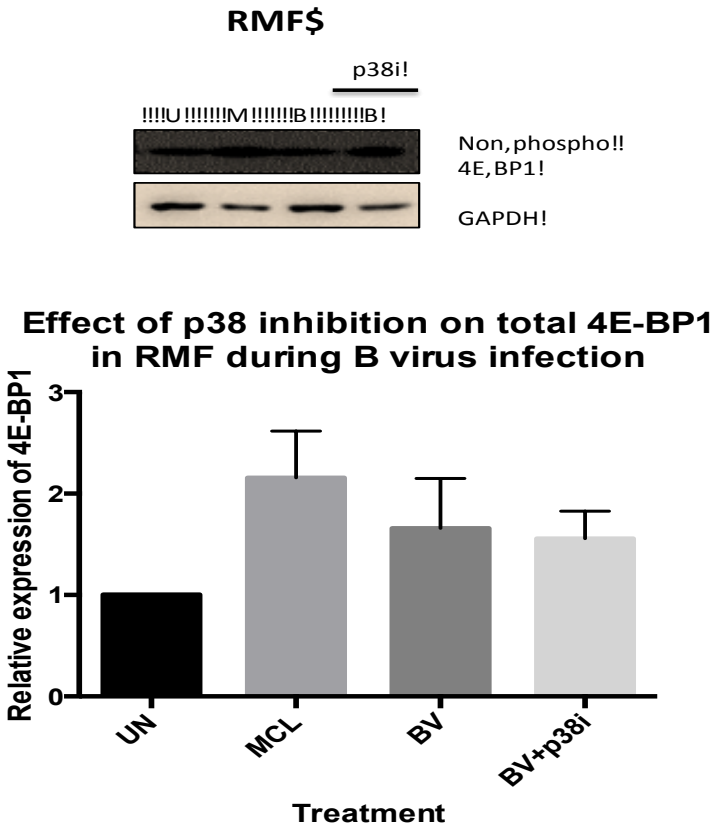

Figure 8: Total 4E-BP1 is not regulated by p38 during B virus infection

Figure 8. Total levels of 4E-BP1 are not regulated by p38 during B virus infection in RMF. (Top) Western blot analysis of HFF and RMF, uninfected (U), treated with mock cell lysate (M), infected with B virus at MOI 5 (B), or infected with BV and treated with SB203580 (p38i) for 24 hours. (Bottom) Densitometric analysis of phospho-4E-BP1 (S65) normalized to the amount of beta tubulin in each sample. Figures are representative of three independent experiments in duplicate.

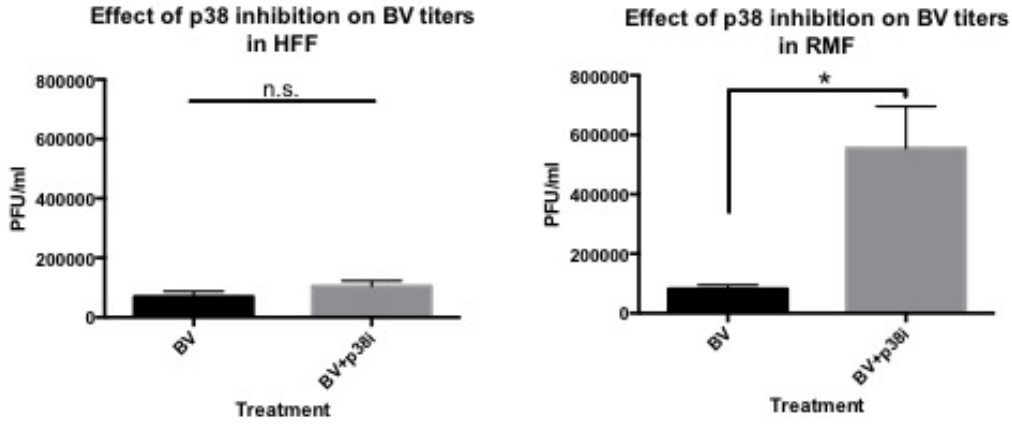

Figure 9: Active p38 regulates B virus titers in a host-dependent manner.

Figure 9: Active p38 regulates B virus titers in a host-dependent manner. Cells were pretreated for 1 hour prior to infection with SB203580. After pretreatment cells were infected with either BV only or BV in the presence of SB203580 (p38i) for 24 hours. Samples were then scraped and collected. Serial dilutions were made of these samples and used to infect VERO cells in duplicate. After 48 hours virus was removed, cells were fixed with methanol and stained with crystal violet. Plaques were counted and PFU/ml was determined. Figures are representative of two independent experiments in duplicate.

3 AIM II: TEST THE HYPOTHESIS THAT INTERACTIONS BETWEEN B VIRUS PROTEIN ICP6 AND US3 WITH CELLULAR TRANSLATION PROTEINS ARE REGULATED BY THE MAPK P38 STRESS PATHWAY DURING B VIRUS INFECTION

In the previous aim we explored the virus:host species interactions between p38 and cellular translation during B virus infection in macaque and human cells representative of those encountered at the time of virus entry. Our data demonstrated that although p38 plays a role in virus replication in the natural host, this role does not appear to be related to regulation of the translation initiation factor eIF4E phosphorylation. This latter regulation appears to rely on other processes that occur during infection. Interestingly, we discovered that the negative regulator of eIF4E, 4E-BP1, is in fact regulated by p38 in macaque fibroblasts under our experimental conditions. As a negative regulator 4E-BP1 binds to eIF4E and prevents its association with eIF4G, thus blocking its phosphorylation by cellular protein MNK. This prevents eIF4E activation and subsequently, mRNA translation. Cellular 4E-BP1 becomes inactive when it is hyperphosphorylated by mTOR, leaving eIF4E to be phosphorylated and activated by MNK and allowing translation to commence. During B virus infection of macaque fibroblasts, we see a significant increase of 4E-BP1 hyperphosphorylation, presumably to allow for translation of viral mRNA to occur unhindered. When p38 activation is inhibited during B virus infection, this increase in hyperphosphorylation fails to occur indicating that p38 plays a critical role in causing the hyperphosphorylation of 4E-BP1 during B virus infection in macaque fibroblasts.

To determine what causes the decrease in hyperphosphorylation of 4E-BP1 in RMF during B virus infection in the absence of the MAPK p38 stress pathway, we considered three possible scenarios: 1) The positive regulator of 4E-BP1, PPM1G, is being upregulated in the

absence of p38; 2) The negative regulator of 4E-BP1, mTOR is being downregulated in the absence of p38; 3) Viral proteins capable of interacting with cellular translational machinery are overriding the cellular machinery resulting in increased viral replication to facilitate infection. These scenarios will each be investigated in this second Specific Aim of the dissertation.

Protein Phosphatase 1G (PPM1G) is a member of the PP2C phosphatase family and removes phosphate groups from serine and threonine residues on proteins. This can be an activation or inactivation modification. Certain members of the PP2C family are negative regulators of cellular stress response pathways. PPM1G dephosphorylates pre-mRNA splicing factors and allowing the formation of the spliceosome. *In vivo* studies also suggest that PPM1G may play a role in regulating cell cycle progression. Furthermore, PPM1G has been shown to dephosphorylate multiple phosphorylation sites of 4E-BP1, thereby regulating cap-dependent cellular translation [59].

Mammalian target of rapamycin (mTOR) was first discovered in 1994 [60]. The mammalian genome produces one TOR protein that has a high molecular weight and has several structural domains that are conserved [58]. mTOR can be autophosphorylated by its own serine/threonine kinase activity and regulates protein synthesis via regulation of 4E-BP1 and S6 kinase (S6K1) phosphorylation [61, 62]. mTOR is regulated by nutrients and growth factors and controls many processes including, but not limited to cellular translational control, cell growth, and proliferation [58]. The translational repressor 4E-BP1 is a well known target of mTOR phosphorylation. By phosphorylating 4E-BP1, mTOR inactivates it causing it to release eIF4E. Once released, eIF4E can be phosphorylated and assemble into the initiation complex, which is a crucial step in cap-dependent translation. Other targets consist of proteins that are important for translation, in particular those that are responsible for recruiting ribosomes to mRNA.

mTOR is also responsible for phosphorylation of eukaryotic initiation factor 4G 1 (eIF4GI). eIF4G contains two groups of phosphorylation sites-one near the N-terminus [63] and the other near the C-terminus, though it is unclear what stimulates the phosphorylation at the N-terminus. The phosphorylation sites near the C-terminus is sensitive to mTOR inhibitors [64]. Because mTOR regulates the phosphorylation of three important proteins in the cellular translational process, we analyzed whether its activity in response to B virus infection may be related to our observations regarding the regulation of eIF4E.

While mTOR and PPM1G are both cellular proteins capable of modifying other cellular proteins that regulate translation, there are also viral proteins of herpesviruses that are known to regulate use of cellular translational machinery.

Three classes of herpes simplex virus genes are known to be expressed during the virus replication cycle and these are divided into each class by the kinetics and regulation of their expression [65]. The first class to be expressed, or the alpha class, is the group of immediate early (IE) genes. Their expression is dependent on the viral protein alpha-transinducing factor (TIF) or viral protein 16 (VP16) that interacts with the cellular transcription factor Oct-1 as well as other cellular proteins to drive transcription [66]. The next class to be expressed is the beta class or the early (E) genes. These genes require the presence of some of the IE proteins for expression. The third class, or the gamma class, are the Late (L) genes that are expressed after the viral DNA has been replicated. This last group can be further divided into groups that either require DNA synthesis for expression or that do not. Some of these late genes are actually expressed throughout the viral replication cycle while others are restricted to the final stages of replication. These classes of viral genes have been described in great detail over the course of

many studies in many different laboratories and have been reviewed in depth by Roizman and colleagues [67].

HSV1, perhaps the most studied member of the alphaherpesvirus simplex viruses of which B virus is also a member, produces two viral proteins that have been shown to play intricate roles in the regulation of translation via manipulation of eIF4E and 4E-BP1. Infected cell protein (ICP) 6, encoded by the gene UL39, of HSV1 has been shown to associate with eIF4G, a scaffolding protein responsible for bringing eIF4E and MNK1 in close proximity. This promotes eIF4F complex formation [68].

The gene UL39 encodes the large subunit of the ICP6, which is a ribonucleotide reductase. The function of this protein is to reduce ribonucleotides to deoxyribonucleotides that can then be incorporated into growing DNA strands. Because of its function, ICP6 is very important for viral DNA synthesis, neurovirulence and reactivation as demonstrated in several animal models and countless studies [66, 69, 70].

In 2006 Walsh and colleagues it was discovered that HSV1 ICP6 was discovered to be an eIF4F assembly chaperone that induced and enhanced complex formation necessary to drive translational processes [68]. Because of the role of ICP6 in eIF4F complex formation in HSV1 infection, we designed experiments to investigate whether or not the role B virus ICP6 within its natural host and how this role may be manipulated in the absence of p38.

A second viral protein of particular interest to us with respect to virus:host interactions at the translational level is the protein encoded by the US3 gene. This gene encodes a protein kinase that forms a homodimer in both HSV1 and HSV2, though their molecular weights are different. US3 does not require any effectors for activity and transfers phosphate groups only from ATP, rather than GTP to serine and threonine residues [71]. US3 also has

autophosphorylation capabilities. Previous work suggests that the protein kinase activity of US3 is encoded by the open reading frame [72, 73]. During HSV1 infection, US3 is responsible for phosphorylating the viral protein encoded by UL34, which is an abundant nonglycosylated virion protein that is likely a type II membrane protein. Reynolds and colleagues revealed that this protein is localized within the nuclear membrane and is necessary for packing progeny virus into envelopes [67, 74]

The US3 of HSV1 has been shown to act as an Akt-like protein that activates mTOR, which in turn affects the activation or deactivation of 4E-BP1. The US3 also phosphorylates the tuberous sclerosis complex 2 (TSC2) on the same residues that are phosphorylated by Akt. This phosphorylation of TSC2 inactivates the protein, which prevents it from inactivating mTOR, thus making mTOR constitutively active [75]. HSV1 US3 also inactivates Akt by dampening activity of viral proteins VP11 and VP12 [76]. Because US3 plays an important role in regulating translational processes during HSV1 infection, we targeted B virus US3 within the natural host to investigate its potential role in regulation of cellular translational machinery. The work proposed in this aim was designed to determine if ICP6 and/or US3 present in B virus had conserved functions with counterparts in HSV1 and whether or not these functions were dependent upon which host was infected by B virus. Arguably, we could assume that at least within the macaque host cells the functions of these homologues are conserved. To begin, we transfected CHO cells to express each of these viral proteins. This was done to verify that our plasmids were producing whole and functional protein product and that p38 inhibition did not interfere with the transfection, nor did either of these transfected proteins compromise the MAPK p38 stress pathways. From there we moved into a more cell relevant model using rhesus

macaque fibroblasts (RMF) as representatives of cells infected during primary infection events within the natural host.

3.1 PPM1G activity is not induced by B virus activation of MAPK p38

As with any biological process, there are checks and balances that regulate protein expression and activation. In the case of 4E-BP1 mTOR functions as a negative regulator wherein it adds phosphate groups to the protein to inactivate it thus 4E-BP1 also requires a positive regulator. The positive regulator of 4E-BP1 is the protein phosphatase PPM1G [59].

As previously discussed, PPM1G belongs to a group of phosphatases that remove phosphates from serine and threonine residues. This group can be divided into two families: the phosphoprotein phosphatases (PPP) and the metal-dependent protein phosphatases (PPM). PPM1G belongs to the PPM family and has been shown to promote the association of 4E-BP1 with the eIF4F cap complex through the dephosphorylation of specific residues of 4E-BP1 [59].

Because of the role that PPM1G plays in 4E-BP1 regulation, it was necessary to identify if p38 inhibition had any affect on its expression and if so, to what extent and whether or not it was in a host-dependent manner. To investigate this question, we performed our inhibition assay followed by western blot and densitometric analyses (**FIG 10**).

Our results indicate that PPM1G was expressed at basal levels in the untreated and MCL treated RMF. This basal level of expression indicates that PPM1G plays a role in the normal everyday function of the cell to regulate translation of mRNAs. When B virus (MOI 5) was used to infect RMF, no substantial changes were observed in PPM1G expression suggesting that PPM1G was not activated in response to stress pathway activation. When p38 activity is ablated during exposure to B virus, again we did not observe a significant modification of PPM1G expression. The stability of PPM1G expression in the presence of virus, as well as in the absence

of p38 activity, suggests that its activity occurs independently of the cellular stress responses associated with B virus activation of the MAPK p38 stress response. From these data, we concluded that the observed p38-dependent decrease in phosphorylation of 4E-BP1 was not attributable to the upregulation of PPM1G to remove the inactivating phosphate groups. Because PPM1G activity was not altered by the inhibition of p38, we decided to further investigate the activity of mTOR during B virus infection as it also plays a role in the regulation of 4E-BP1 and has been shown to be susceptible to being regulated by viral proteins.

3.2 mTOR activity is unaffected by B virus activation of MAPK p38

As the negative regulator of 4E-BP1, mTOR adds phosphate groups to the different phosphorylation sites on 4E-BP1. This hyperphosphorylation via mTOR prevents 4E-BP1 from attaching to eIF4E and acting as an inhibitory regulator to cellular translation. Not only does mTOR phosphorylate 4E-BP1, but it has also been shown to phosphorylate other translational proteins such as eIF4G and eIF4B, both of which play crucial roles in the initiation phase of translation [58]. Because mTOR mediates phosphorylation of several factors involved in the initiation phase of translation, we next considered how B virus activation of MAPK p38 stress pathways affect mTOR. To experimentally evaluate our consideration, we collected samples from our inhibition assays and performed western blots and densitometric analyses to measure relative levels of mTOR phosphorylation attributable to the activation of MAPK p38 stress response (**FIG 11**).

Our results confirmed that in untreated and MCL treated cells, mTOR was activated at basal levels. Because this protein regulates several processes within the cell, this was predictable. Our data revealed that B virus did not increase or decrease mTOR phosphorylation and

activation beyond apparent basal levels found in unmanipulated cells. These findings strongly suggested that B virus does not regulate the mTOR pathway, but the question remained that perhaps B virus exploits the pathway for efficient viral replication and spread thus the pathways remains unperturbed by the infection. When p38 phosphorylation was inhibited, we observed no significant changes in the levels of phosphorylated mTOR. It is important to note that once phosphorylated, mTOR can be activated by stimulants to do a variety of tasks including phosphorylation of 4E-BP1. Some of these stimulants require basal levels of p38 phosphorylation to stimulate mTOR to phosphorylate 4E-BP1 [77]. This could explain our observed decrease in levels of phosphorylated 4E-BP1 in RMF cells treated with the inhibitor of MAPK p38 phosphorylation. Together, our data indicate that mTOR can generally phosphorylate n4E-BP1 independently of the MAPK p38 stress pathway.

3.3 ICP6 is unnecessary to enhance eIF4F activity in the presence of p38.

In HSV1, Walsh and colleagues determined that ICP6, which is a ribonucleotide reductase, interacts with eIF4G to enhance viral protein production during infection. In HSV1 infected cells ICP6 acts as an eIF4F-assembly chaperone and is the first example of a protein that promotes eIF4F complex formation during viral infection [68]. From our previous work sequencing the complete B virus genome, we identified ORFs present in the B virus genome. Of those, UL39 and UL40 were predicted to encode the large and small subunit of the ribonucleotide reductase, respectively. B virus ICP6 shares approximately 71% identity with HSV1 ICP6 and approximately 73% with HSV2 ICP6, though similarity is slightly higher at 77% and 78%, respectively [4]. Because of the high rate of similarity, we hypothesized that the ribonucleotide reductase in B virus had similar functions to that of HSV1. Using this information we next planned experiments to determine if ICP6 is capable of regulating eIF4F complex in the

presence or absence of p38 in RMF [78]. To investigate the role of B virus ICP6, we inserted the UL39 gene into a pcDNA vector and transiently transfected Chinese hamster ovarian (CHO) cells in the presence or absence of p38. We then collected whole cell lysate and conducted Western blot and densitometric analyses to observe phosphorylation and activation of eukaryotic initiation factors that are members of the eIF4F complex. To begin, we first needed to verify the successful transfection of UL39 into CHO cells (**FIG 12**). We verified successful protein production by detection of the V5 epitope present within the vector.

Because we were uncertain of how efficient the transfection would be, we transfected cells with either two or four micrograms of plasmid DNA and compared the levels of V5 epitope expression. In cells transfected with two micrograms, we observed thick dark bands corresponding to B virus ICP6 with the expected molecular weight of approximately 115.7 kD. There is also an equally dark band directly below our ICP6 band as well as a few much lighter bands. These bands are likely generated from degradation of the translated protein as well as some non-specific binding of the anti-V5 antibody in the case of the lighter bands. Alternatively, phosphorylation at multiple sites may have resulted in products of varied molecular weight of the protein. The same bands were seen in western blots prepared from cells transfected with four micrograms; however, expression of the V5 epitope was verified in both groups, validating that we were able to successfully transfect CHO cells with our UL39 plasmid. In the untransfected cells, designated in the figure as “Un”, we see no detectable V5 epitope. These cells were used as a negative control.

With our transfection assay yielding predicted outcomes, our next step was to inhibit p38 activity while simultaneously transfecting these CHO cells to validate that cells remained healthy (**FIG 13**). Because we had efficient transfection yields with transfecting only 2 μg of DNA, we

standardized transfections using 2 μg from this experiment forward. When CHO cells are transfected with 2 μg of plasmid DNA, we observed a large dark band appearing at approximately 115 kD as was previously noted. In conjunction with V5, we also see expression of phosphorylated p38. In untransfected cells, we observed neither the expression of V5 nor p38. Expression of ICP6 was sufficient to activate p38. In CHO cells treated with the p38 inhibitor, we readily detected the V5 epitope of the fusion peptide validating B virus ICP6 protein was produced. We also saw significant reduction in p38 phosphorylation. In the untransfected cells treated with p38 inhibitor, we observed neither V5-fusion protein expression nor p38 phosphorylation. These data reveal that ICP6 alone is capable of inducing p38 phosphorylation in CHO cells and that transfection and simultaneous inhibition of p38 phosphorylation are achievable in this cell line.

To determine whether or not ICP6 was capable of affecting the assembly of the translation initiation comples in the presence or absence of the MAPK p38 stress pathway activation, we transfected with our plasmid bearing ICP6 into cells with functional MAPK p38 stress pathways (**FIG 14**). Results of this experiment revealed expression of eIF4G phosphorylation. Investigators have reported that phosphorylation of eIF4G is not necessarily indicative of activation of function, but this modification may cause a conformational change in the protein that affects its activity [79]. In cells that were not transfected with ICP6, we see very similar levels of eIF4G phosphorylation, suggesting that B virus ICP6 does not enhance the phosphorylation of eIF4G. When the stress pathway was inhibited as described, the levels of eIF4G phosphorylation remained unchanged validating that p38 activity is not necessary for eIF4G phosphorylation. When the phosphorylation status of eIF4E was explored, very interesting results were observed. In untransfected cells, we observed basal levels of eIF4E

phosphorylation indicating a normal level of activation. When B virus ICP6 was transfected into these cells, we observed a marked decrease in eIF4E phosphorylation. This was in direct contrast to what we expected to see based on the fact that HSV1 ICP6 was reported to enhance the phosphorylation of eIF4E during infection. Inhibition of the MAPK p38 stress pathway revealed that levels of basal activity observed in untransfected cells decreased when compared to the untransfected cells in the presence of p38. The levels of eIF4E phosphorylation in cells transfected with B virus ICP6 were independent of the functional stress pathways. Together, these data suggested that B virus ICP6 appeared to have the opposite role of the HSV1 ICP6 homolog, causing a decrease in phosphorylation of eIF4E, which would be predicted to cause a decrease in the rate at which translation occurs within the cell. Because these results were obtained in CHO cells, which are not representative cell populations of primary infection sites in the natural host, the transfection assays were subsequently performed in the RMF cell line in order to confirm that B virus ICP6 behaves the same way in the RMF as it does in the CHO cells or to show that ICP6 acts differently depending on the cell type.

3.4 B virus US3 requires the presence of phosphorylated p38 to stimulate mTOR and, in turn, 4E-BP1 inactivation.

Once we examined the role of ICP6 in the presence and absence of p38 activity, we focused on discerning the effects of p38 on B virus US3. Investigators have reported that HSV1 US3 behaves like the cellular protein kinase Akt and phosphorylates several of its substrates including mTOR, yet shares little or no sequence homology with Akt. Data suggests that HSV1 US3 activates mTOR by phosphorylating and inactivating TSC2, which is an inhibitory molecule to mTOR activitation. With TSC2 inactivated, mTOR can now be constitutively active and can phosphorylate its downstream effectors 4E-BP1 and S6K to enhance translational activity. B

virus US shares approximately 58% identity with HSV1 ICP6 and approximately 57% with HSV2 ICP6, though similarity is slightly higher at 70% and 65%, respectively. Even though the similarities between BV US3 and HSV1 and 2 US3 are not very high, it was still important to determine what effect, if any BV US3 has on this particular pathway as this protein in B virus has not been previously studied. To analyze the effect of B virus US3 in the presence and absence of p38, we expressed B virus US3 in CHO cells and simultaneously inhibited p38 phosphorylation. We analyzed the whole cell lysate samples collected using western blot and subsequent densitometric analyses. As before, we began with a CHO cell model to optimize our transfection and inhibition assay and from there, moved into a more relevant cell model using the RMFs to analyze the effects of US3 in the presence and absence of p38 activity.

To begin, we first analyzed the efficiency of transfection of B virus US3 into CHO cells (**FIG 15**). Again we transfected with two or four micrograms of plasmid containing the B virus US3 insert to determine which concentration of DNA was optimal and analyzed expression of the V5 epitope of the expressed fusion protein. We observed two dense bands in both the two and four microgram transfected cells correlating with our expected molecular weight of approximately 55.5 kD. The doublet bands were to be expected as this protein forms homodimers. Other lighter and smaller bands were also observed at lower molecular weights. These could be the degradation from the expressed protein or non-specific anti-V5 antibody binding. Our data showed that US3 was successfully transfected into CHO cells. Our next step was to analyze transfection of US3 in the presence and absence of p38 in CHO cells (**FIG 16**).

Our results showed that in cells transfected with B virus US3, p38 phosphorylation was apparent indicating that the presence US3 resulted in phosphorylation and activation of p38. In our vector-transfected cells, we see neither the V5-tagged fusion protein nor p38 phosphorylation

and the same is true when the untransfected cells were analyzed. These observations suggest that in the absence of B virus US3, p38 is not phosphorylated which suggests the cell remains unstressed, or during stress this viral protein may block the activation of the pathway. When p38 activity is inhibited, US3 expression is observed, but phosphorylation of p38 is significantly reduced. This indicated to us that the inhibition was successful and did not interfere with expression of US3 from the vector. In the absence of p38, the vector-transfected and untransfected cells showed neither V5-tagged fusion protein expression nor p38 phosphorylation. Once we optimized our transfection and simultaneous inhibition assay for the use of plasmids containing US3 in CHO cells, we validated the effects US3 had on mTOR and its downstream effector 4E-BP1 in the presence and absence of p38 activity.

The first protein we analyzed was mTOR phosphorylation (**FIG 17**). In US3 transfected cells we observed phosphorylated mTOR relatively increased when compared to the vector-transfected and the untransfected cells. This observation is what we predicted as we know that HSV1 US3 is capable of phosphorylating mTOR and inducing its activity. When p38 activity was ablated, we did not observe upregulation of mTOR phosphorylation noted to occur in the presence of p38. Thus B virus US3 appears to increase levels of phosphorylated mTOR, but this requires activated MAPK p38. The levels of mTOR phosphorylation remained the same in vector-transfected and untransfected cells remained the same in the absence of p38 activity.

Because we know that HSV1 US3 acts like cellular kinase Akt and is capable of downregulating its activity, we examined at the effect of US3 on Akt phosphorylation expression levels and whether or not this was modulated by the presence of p38 (**FIG 18**). From these experiments we observed that in the presence of B virus US3, Akt phosphorylation occurs independently of MAPK p38 stress pathway activation. Phosphorylated Akt levels were apparent

in western blots of US3 transfected cell lysate, but observed levels were comparable to levels seen in empty vector-transfected and untransfected cells. When p38 activity was inhibited, the levels of phosphorylated Akt remained unchanged. This data suggested that B virus US3 did not interfere with phosphorylated Akt levels, despite taking over some of the functions of Akt. These observations provide strong support that B virus US3 does not share at least one function of HSV1 US3, the latter which appears to have diverged with respect to this function. This functional difference could be associated with sequence differences between HSV1 ICP6 and B virus ICP6, or that in CHO cells US3 behaves differently than it would in its natural host.

Lastly, we looked at the effect of US3 on hyperphosphorylation of 4E-BP1 in the presence and absence of phosphorylated p38 (**FIG 19**). In US3 transfected cells, we see hyperphosphorylation of 4E-BP1 levels that are comparable to the vector-transfected and untransfected cells indicating that B virus US3 does not enhance or reduce 4E-BP1 phosphorylation in the presence of functional MAPK p38 stress responses. In the absence of p38, in US3 transfected cells 4E-BP1 phosphorylation is significantly reduced when compared to the empty vector-transfected and untreated cells without p38 activity. These observations provide evidence that the MAPK p38 stress pathway is essential for US3 function for the maintenance of 4E-BP1 hyperphosphorylation, most likely through the activation of mTOR. These findings agree with our initial findings of decreased levels of hyperphosphorylated of 4E-BP1 during B virus infection in the absence of p38 within cells of the natural host. As was stated earlier, because these results were obtained in CHO cells, the transfection assays were then performed using the primary RMF cell line in order to confirm that B virus US3 affects the RMF cells as it does in the CHO cells or to show that US3 has different effects depending on the cell species in which it is expressed.

3.5 Transfection of primary RMF cells with BV ICP6 and US3

In previous experiments we have shown the effects of the presence of BV ICP6 and US3 in Chinese hamster ovarian (CHO) cells; however, these results may differ when these virus proteins are expressed in cells from the natural host of B virus infection. For this reason, we attempted to transfect RMF with either ICP6 or US3 in the presence or absence of p38 activity to determine whether or not either or both viral proteins affected stress pathway function or required intact stress pathways for their individual functions. Primary cell transfection can be more challenging than transfecting immortalized cell lines like CHO cells. The reasons for this are unclear though differences in rates of transfection and tolerances of cytotoxic transfection reagents may contribute. Typically primary cells used in transfection assays are embryonic or stem cells. Our RMF cell line is isolated from macaques of different ages, most being adult animals. Successful isolation and growth of fibroblasts from adult macaques is challenging and because the cells are much older than the embryonic or stem cells typically used for transfection. To begin, we performed the same transfection assay used in the CHO cell line in the RMF cell line as described in the section Materials and Methods. This experiment failed to yield transfected cells and it was determined that rate of transfection was the probable cause. To optimize the transfection of RMF alone, we transfected cells as previously stated but without use of the MAPK p38 inhibitor SB203580 and instead of a 24-hour transfection period, we allowed the transfections to proceed for 48 hours and 72 hours for each protein. We found that B virus ICP6 requires 48 hours for transfection and expression of the protein in RMF (**FIG 20**) and that US3 requires 72 hours for transfection and expression of the protein in RMF (**FIG 21**). We also observed that unlike ICP6 and US3 expression in CHO cells, there were not multiple bands appearing in each sample set and no doublets, thus processing of these viral proteins in macaque

cells was likely different than that observed in transfected CHO cells. There is only one non-specific band that appeared at approximately 37 kDa, which was also observed in untransfected cells indicating that the anti-V5 antibody cross-reacted with a cellular protein (data not shown). These results were encouraging considering the already difficult task of transfecting primary cells.

Thereafter we first analyzed the effects of expressed ICP6 and US3 in RMF cells on the cellular proteins that may be activated or repressed by these viral proteins. Based on our optimization of ICP6 and US3 transfection, we transfected RMF cells with 2 μg of either UL39 (ICP6) pcDNA or US3 pcDNA for 48 hours and 72 hours, respectively (**FIG 22)**. We collected the whole cell lysate and analyzed an aliquot of this to determine relative levels of phosphorylated eIF4F complex members as well as phosphorylated mTOR. Phosphorylation of eIF4G, the scaffolding protein, was markedly decreased in transfected RMF, which is different from what is seen in UL39 transfected CHO cells; however, expression of phosphorylated eIF4G was also low in empty vector transfected RMF indicating that the transfection process itself was likely responsible for the decrease in eIF4G activation, perhaps due to the stress of transfection and not necessarily the presence of ICP6 (**FIG 23**). Despite the fact that eIF4G requires phosphorylation to be active, its activation does not correlate to the activation of eIF4E therefore, the transfection of UL39 may influence eIF4G phosphorylation independently of eIF4G activation. In UL39 transfected RMF, we observed a marked decrease also in eIF4E phosphorylation, which contradicts the observation of ICP6 activation of eIF4E in HSV1 infected cells, but is similar to the results seen in UL39 transfected CHO cells [68] (**FIG 24**). Together, these results confirmed our observation in transfected CHO cells and thus suggested that the effects of ICP6 observed in CHO cells is indicative of its true nature in its natural host.

Phosphorylation of mTOR, the inactivator of 4E-BP1, appeared greatly decreased in US3 transfected RMF (**FIG 25**). It is interesting to note that the RMF transfected with empty vector expressed even less phosphorylated mTOR, again indicating that the decrease in mTOR activation may be due to stress caused by the transfection process. These results are again opposite of those observed in transfected CHO cells expressing mTOR; however, this difference in mTOR activation may be due to stress as evidenced by the decreased in mTOR activation in cells only transfected with empty vector pcDNA as seen in the previous experiment described.

We next designed experiments so that we could determine how expression of B virus ICP6 or US3 affected p38 activity and expression of cellular proteins. This task proved to be challenging due to what we attributed to the putative toxicity of the transfection reagents as well as the MAPK p38 inhibitor. The Lipofectamine™ reagents used in the transfection were toxic to the cells, but have been optimized by the company to minimize damage to the cells and maximize transfection; however, this optimization was done in established cell lines that are susceptible to transfection, not in primary cell lines. In RMF transfected with UL39 or US3 there were cytotoxic effects. Some cells had detached from the plate and were floating in the medium. Those that remained adhered to the plates were producing stress granules and significant cell rounding was observed. RMF transfected in the presence of SB203580 also had detached cells in the medium. To quantify the cytotoxicity of the transfection reagents and SB203580, we performed a Trypan blue exclusion assay to determine the ratio of dead cells to live ones. In RMF transfected with UL39, approximately 75% of cells are dead while 25% remain alive. In RMF transfected with empty vector, approximately 68% of cells are dead. In untransfected cells, we see that approximately 80% of cells are alive, indicating that the transfection reagents are causing significant cell death. When SB203580 is added to the transfection assay, the results are

similar, with slightly more cell death at approximately 80% of the total population (**FIG 26**). In RMF transfected with US3, we see approximately 65% of the total cell population is dead with a remaining 35% of cells still alive. In RMF transfected with empty vector, we see approximatel 55% cell death and in untransfected cells, the majority of cells are alive at approximately 95% of the total cell population. In cells transfected with US3 in the presence of SB203580, we see approximately 72% of the total cell population is dead with 28% remaining alive. The empty vector transfected cells yield similar numbers while 90% of the total cell population of untransfected cells remains alive (**FIG 27)**. Together, these data indicate that the Lipofectamine transfection reagents are causing significant cell death in RMF and although transfection is somewhat successful as indicated previously, the amount of stress induced within the cells masks any effect produced by the presence of the viral proteins in RMF. To further demonstrate the cytotoxicity of the transfection reagents to RMF, pictures of the cells under the various conditions were taken microscopically. In the control group, which is RMF in DMEM containing 10% FBS, we see the typical morphology associated with this cell type. The cells are elongated at the ends and become thicker toward the center where the nucleus is housed. In the untransfected group, which is RMF in Opti-MEM as suggested by Lipofectamine transfection protocol, cell structure looks slightly different with cells appearing rounder in shape as opposed to elongated, but still no evidence of stress or dead cells as there are no cells floating in the medium. In the vector group, which is RMF transfected with empty vectors, we start to see signs of cytotoxicity such as cell rounding and cells floating. The results are the same with the addition of SB203580 (**FIG 28**). RMF transfected with either UL39 or US3 show tremendous cytotoxic effects with symptoms such as detachment from the surface of the plate, cell rounding, and the production of stress granules, regardless of whether or not SB203580 is present (**FIG 29)**. To

reduce the cytotoxicity, we reduced the amount of Lipofectamine reagents used to half of the recommended amounts. With reduced amounts of the transfection reagents the cells appeared healthier and there were less cells floating in the medium, but when the transfection was verified there was neither ICP6 nor US3 expressed (not shown). It was determined that simultaneous transfection and inhibition of p38 via SB203580 was not possible due to the fact that RMF are primary cells that are much older than primary cells usually used in transfection assays and that the sensitivity of the RMFs to the transfection reagents left the majority of cells dead. Alternatives to the use of SB203580 as an inhibition method will be discussed in the conclusions section of this aim.

3.6 Aim II Conclusions

The experimental questions that were explored in this second aim demonstrated four main points. First, that pcDNA vectors containing genes for B virus proteins ICP6 and US3 can be transfected into CHO cells to produce in tact, and functional proteins. Although CHO cells do not represent primary infection in the natural host or even a foreign host that is often exposed to B virus, this was a step forward in B virus research to begin determining the function of these important viral proteins during the course of infection and whether or not they have differing effects based on the host they occupy. Second, we showed that in CHO cells, B virus ICP6 does not enhance activation of the eIF4F complex. These results are in direct contrast to our expected findings as the ICP6 protein belonging to HSV1 is capable of enhancing activation of this complex. There are two obvious possibilities as to why these results differ from what was expected. The first is that CHO cells are not the natural host of this virus. In HSV1 studies, human cells were used therefore they were studying the effects of this protein within its natural host. The fact that ICP6 behaved differently from expected in CHO cells could be just a product

of the cell type that was transfected. The second possibility is that B virus ICP6 function really does differ from that of HSV1 ICP6 function. In this case, the identification of a difference in function of ICP6 between HSV1 and BV would be a novel finding in the field of herpesvirus research, as it has been assumed that functions of viral proteins are conserved through the families of herpesviruses.

Third, we found that mTOR phosphorylation is significantly enhanced by the presence of B virus US3. This result is in accordance with our expected findings as US3 has been shown to enhance mTOR phosphorylation and activity during HSV1 infection. We also showed that this activation depends on the presence of p38 activity. In the absence of p38, mTOR phosphorylation is greatly decreased even in the presence of US3. This indicates that mTOR phosphorylation does require p38 activity to an extent. Lastly, we found that phosphorylation of 4E-BP1 at serine 65 is maintained in the presence of US3; however, this is also dependent on p38 activity. This result was also expected as 4E-BP1 inactivation is directly affected by mTOR activity. Also, previous studies have shown that p38 activity does stimulate certain functions of mTOR, such as 4E-BP1 phosphorylation, but does not affect mTOR phosphorylation itself.

Though these results are intriguing and may bring novel results to the fields of B virus research and herpesvirus research, it is pertinent that the roles of ICP6 and US3 be examined in the natural host cells. With transfection in the natural host cells, we will be able to answer two questions that could not be answered with only the CHO transfection assays: 1) How do ICP6 and US3 behave in their natural host and 2) Are the functions discovered in CHO cells the same as those in macaque cells. The answers to these questions are of great importance because they will tell us the true nature of these proteins during infection and also, whether or not their function differs depending host cell. This finding could provide great insight into the difference

in outcome of infection between human hosts and macaque hosts and also be a big stepping stone in studying any zoonotic virus infection.

We were unable to perform this last task of transfecting natural host cells as these cells are primary fibroblasts that are isolated by the hands of researchers in our labs. This is significant for two reasons. The first being that most primary cell lines used in transfection assay are embryonic or stem cells, which means that they are heartier and able to withstand the cytotoxic reagents used for lipofection. Because we isolate our fibroblasts from necropsy samples provided to us by another institutions, most of the samples we receive are harvested from animals that are at least one year old, though most of the time they are a bit older. The cell line used in these assays came from an animal that was approximately 4 years old at the time of necropsy. The second reason is that primary cell lines used in transfection assays are commercially available and are purchased from a company that create a homogenous cell line for optimal performance. Because rhesus macaque fibroblasts were not commercially available at the time these experiments were being conducted, we had to isolate fibroblasts ourselves using a hybrid of different isolation techniques to obtain optimal results.

Lipofectamine® Transfection reagents are currently the most referenced transfection reagents. To date it is the most efficient versatile reagent for the widest range of cells including cell types that are difficult to transfect, such as primary cells (78). The fact that we were unable to transfect our primary rhesus macaque fibroblast cell lines using these reagents implies that the cells are not able to withstand the cytotoxicity of the reagents rather than the implication that the reagents aren't compatible for these cells.

3.7 Figures

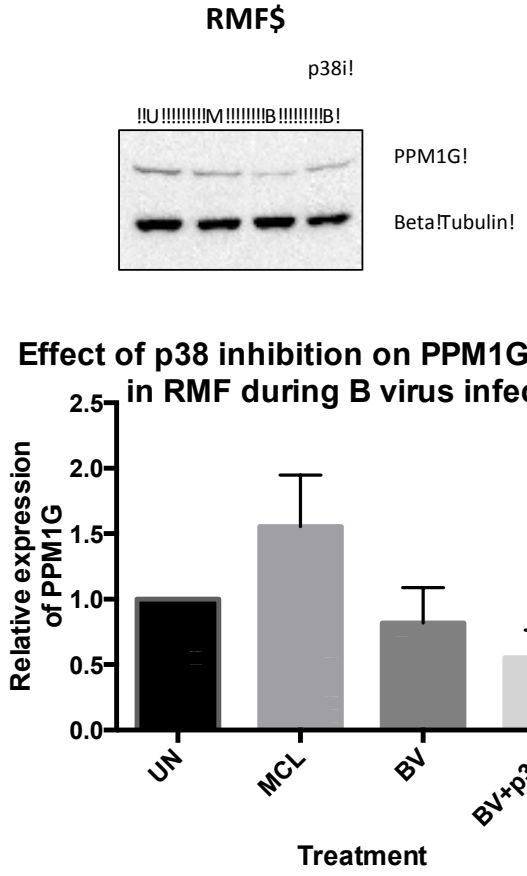

Figure 10: PPM1G is not induced by B virus infection or p38

Figure 10: PPM1G is not induced by B virus infection or p38. (Top) Western blot analysis of RMF uninfected (U), treated with mock cell lysate (M), infected with B virus at MOI 5 (B), or infected with BV and treated with SB203580 (p38i) for 24 hours. (Bottom) Densitometric analysis of PPM1G normalized to the amount of GAPDH in each sample. Figures are representative of three independent experiments in duplicate.

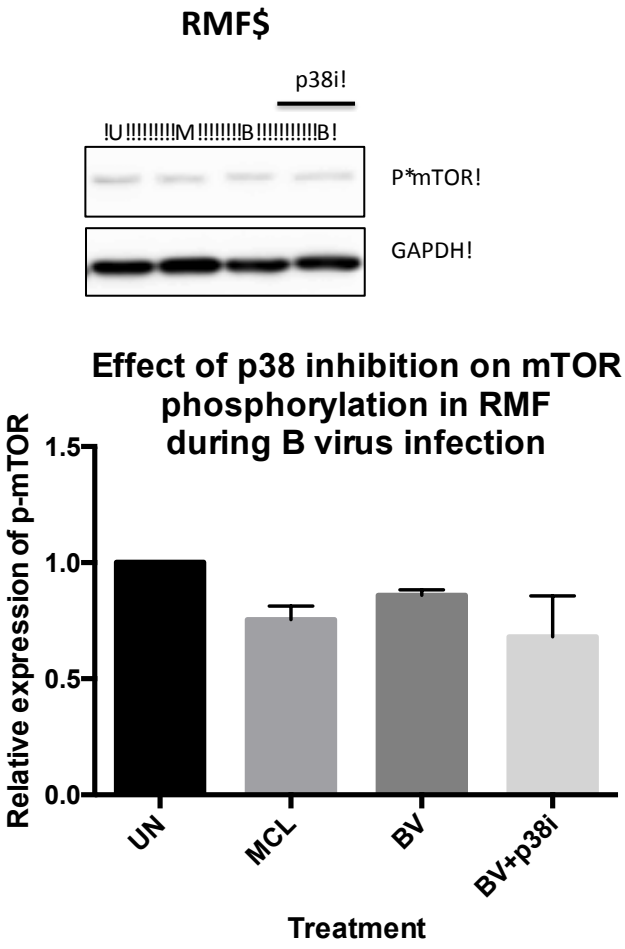

Figure 11: Phosphorylated mTOR is not upregulated by B nor dependent on p38

Figure 11: Phosphorylation of mTOR is not upregulated by B virus and is not dependent on p38. (Top) Western blot analysis of RMF uninfected (U), treated with mock cell lysate (M), infected with B virus at MOI 5 (B), or infected with BV and treated with SB203580 (p38i) for 24 hours. (Bottom) Densitometric analysis of phospho-mTOR normalized to the amount of GAPDH in each sample. Figures are representative of three independent experiments in duplicate.

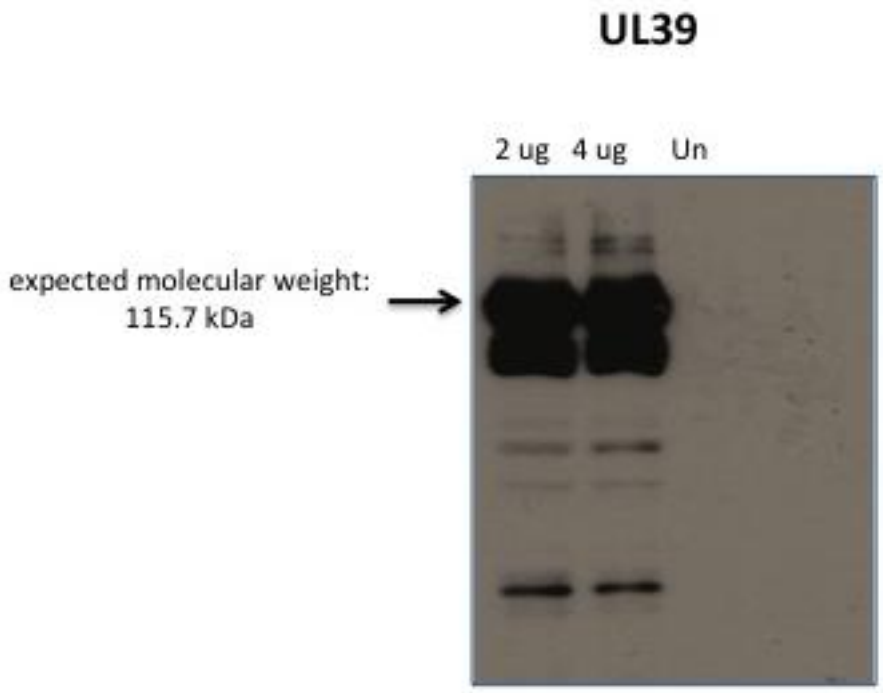

Figure 12: Verification of UL39 transfection in CHO cells

Figure 12: Verification of UL39 transfection in CHO cells.Western blot analysis of CHO cells transfected with 2 ug or 4 ug of plasmid DNA and untranfected CHO cells. Antibodies against the V5 epitope in the plasmid were used to detect protein product. Figures are representative of two independent experiments in duplicate.

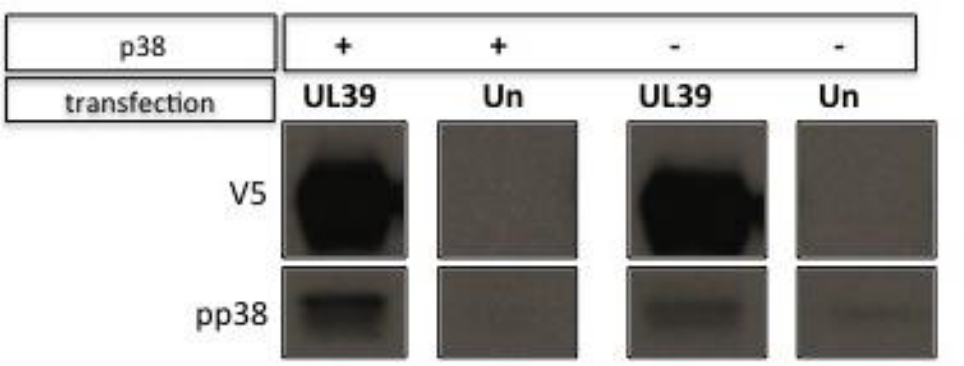

Figure 13: Verification of transfection and p38 inhibition in CHO cells

Figure 13: Verification of transfection and simultaneous p38 inhibition in CHO cells. Western blot analysis of CHO cells transfected with 2 ug of UL39 plasmid DNA or untransfected in the presence or absence of SB203580. Antibodies against the V5 epitope in the plasmids and phospho-p38 were used to detect the presence of the proteins. Figures are representative of two independent experiments in duplicate.

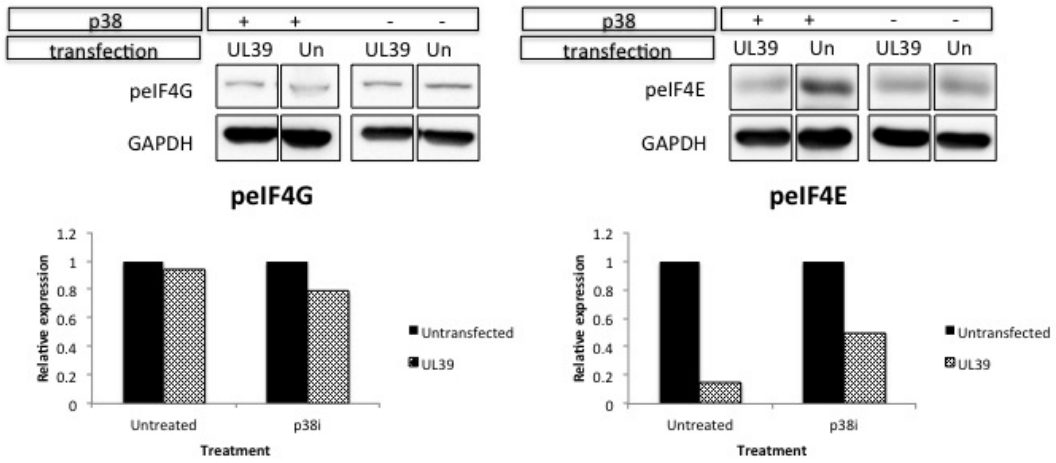

Figure 14: ICP6 does not enhance eIF4G or eIF4E phosphorylation

Figure 14: ICP6 does not enhance eIF4G or eIF4E phosphorylation. (Top) Western blot analysis of CHO cells transfected with 2 ug of UL39 plasmid DNA or untransfected in the presence or absence of SB203580. Antibodies against the V5 epitope in the plasmids and phospho-eIF4G and phosphor-eIF4E were used to detect the presence of the proteins (Bottom) Densitometric analysis of phospho-eIF4G and phosphor-eIF4E normalized to the amount of GAPDH in each sample. Figures are representative of two independent experiments in duplicate.

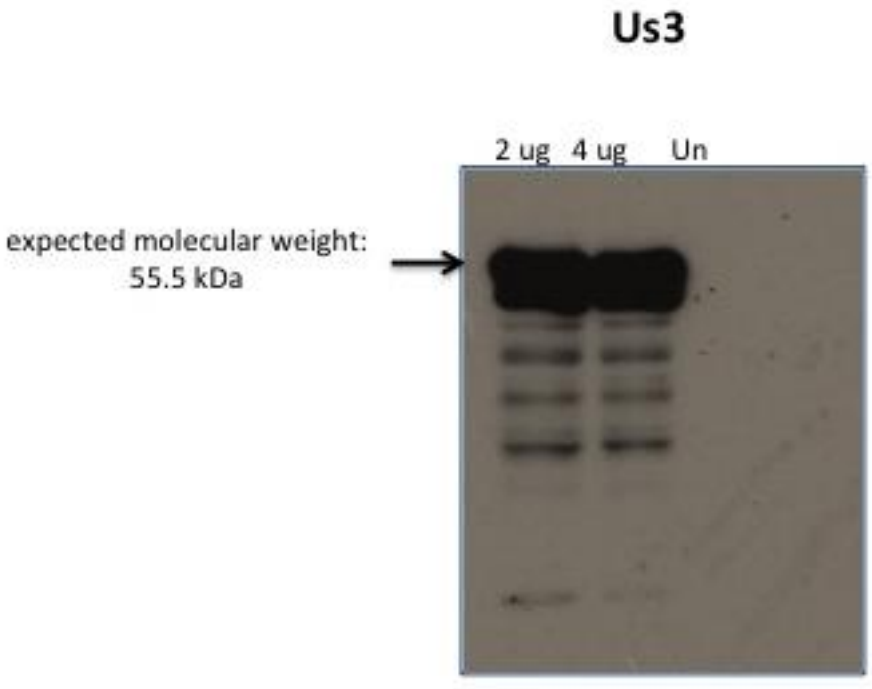

Figure 15: Verification of US3 transfection in CHO cells

Figure 15: Verification of US3 transfection in CHO cells. Western blot analysis of CHO cells transfected with 4 ug or 2 ug of US3 plasmid DNA or untransfected. Antibodies against the V5 epitope in the plasmids were used to detect the presence of the proteins. Figures are representative of two independent experiments in duplicate.

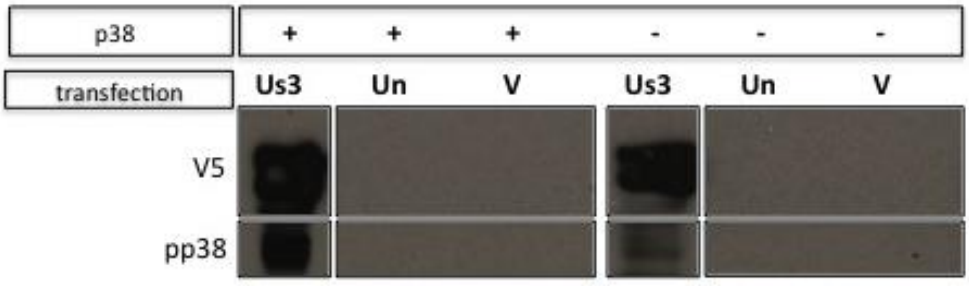

Figure 16: Verification of US3 transfection and p38 inhibition in CHO cells

Figure 16:Verification of US3 transfection and p38 inhibition in CHO cells. Western blot analysis of CHO cells transfected with 2 ug of US3 plasmid DNA or untransfected in the presence or absence of SB203580. Antibodies against the V5 epitope in the plasmids and phospho-p38 were used to detect the presence of the proteins. Figures are representative of two independent experiments in duplicate.

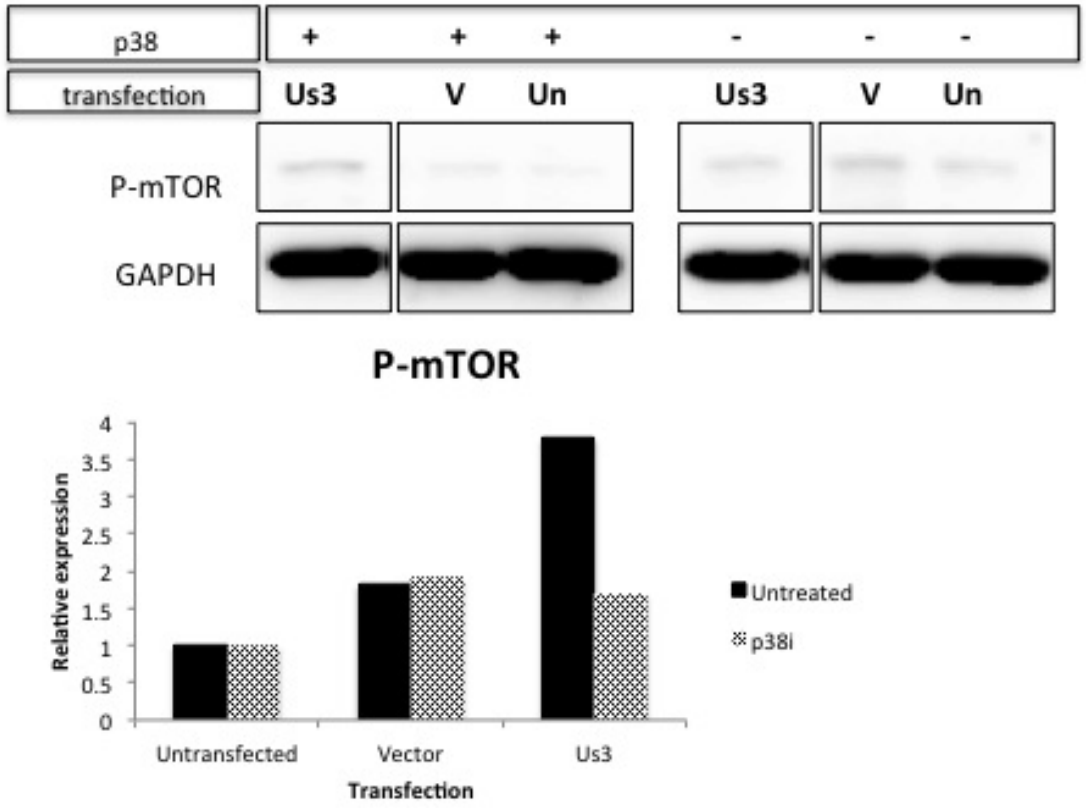

Figure 17: US3 upregulates mTOR phosphorylation in a p38-dependent manner

Figure 17: B virus US3 upregulates mTOR phosphorylation in a p38-dependent manner. (Top) Western blot analysis of CHO cells transfected with 2 ug of US3 plasmid DNA or untransfected in the presence or absence of SB203580. Antibodies against the V5 epitope in the plasmids and phospho-mTOR were used to detect the presence of the proteins (Bottom) Densitometric analysis of phospho-mTOR normalized to the amount of GAPDH in each sample. Figures are representative of two independent experiments in duplicate.

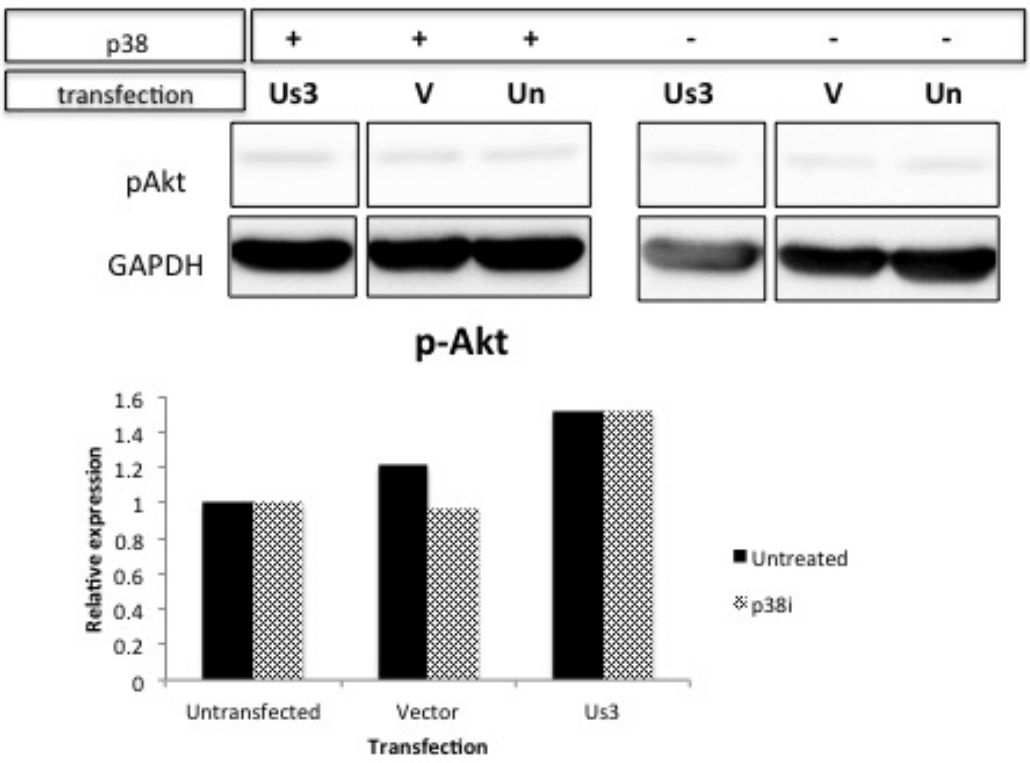

Figure 18: US3 does not affect Akt activity in the presence and absence of p38

Figure 18: US3 does not affect Akt activity in the presence and absence of p38. (Top) Western blot analysis of CHO cells transfected with 2 ug of US3 plasmid DNA or untransfected in the presence or absence of SB203580. Antibodies against the V5 epitope in the plasmids and phospho-Akt were used to detect the presence of the proteins (Bottom) Densitometric analysis of phospho-Akt normalized to the amount of GAPDH in each sample. Figures are representative of two independent experiments in duplicate.

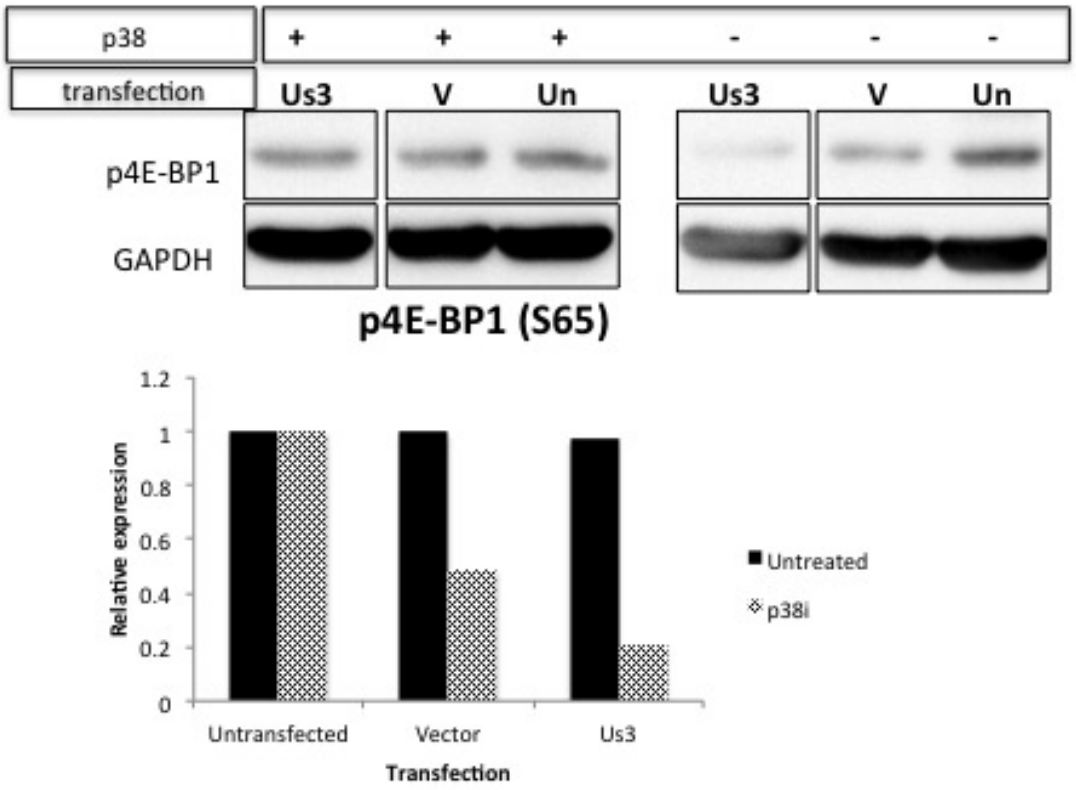

Figure 19: US3 supports 4E-BP1 phosphorylation in a p38-dependent manner

Figure 19:US3 supports 4E-BP1 phosphorylation in a p38-dependent manner. (Top) Western blot analysis of CHO cells transfected with 2 ug of US3 plasmid DNA or untransfected in the presence or absence of SB203580. Antibodies against the V5 epitope in the plasmids and phospho-4E-BP1 (S65) were used to detect the presence of the proteins (Bottom) Densitometric analysis of phospho-4E-BP1 (S65) normalized to the amount of GAPDH in each sample. Figures are representative of two independent experiments in duplicate.

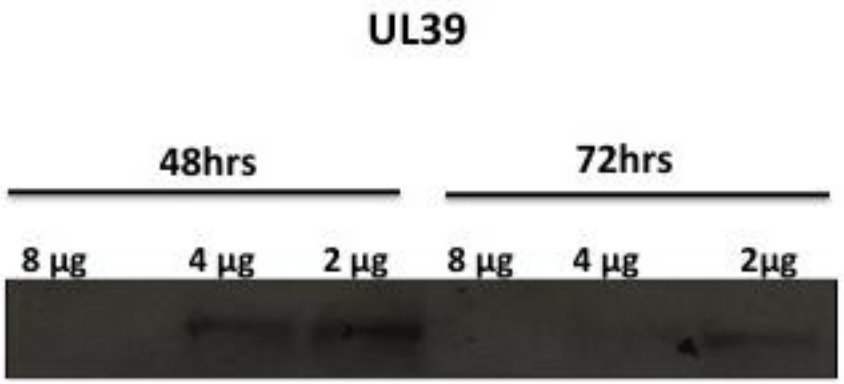

Figure 20: Optimization of UL39 transfection in RMF

Figure 20: Verification of UL39 transfection in RMF. Western blot analysis of RMF transfected with 8 ug, 4 ug or 2 ug of UL39 plasmid DNA. Antibodies against the V5 epitope in the plasmids were used to detect the presence of the proteins. Figures are representative of two independent experiments in duplicate.

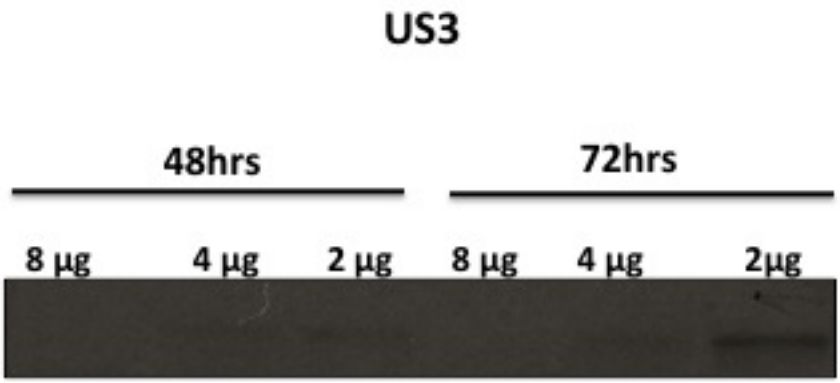

Figure 21: Optimization of US3 transfection in RMF

Figure 21: Verification of US3 transfection in RMF. Western blot analysis of RMF transfected with 8 ug, 4 ug or 2 ug of US3 plasmid DNA. Antibodies against the V5 epitope in the plasmids were used to detect the presence of the proteins.

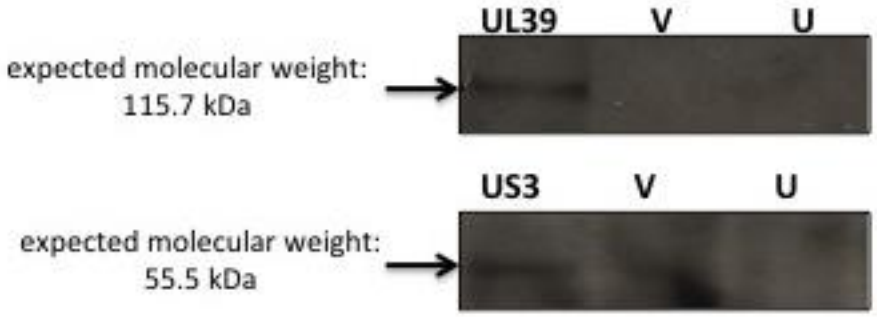

Figure 22: Verification of transfection in RMF

Figure 22: Verification of UL39 and US3 transfection in RMF. Western blot analysis of RMF transfected with 2 ug of UL39 or US3 pcDNA, empty vector or untransfected. Antibodies against the V5 epitope in the plasmids were used to detect the presence of the proteins. Figures are representative of two independent experiments in duplicate.

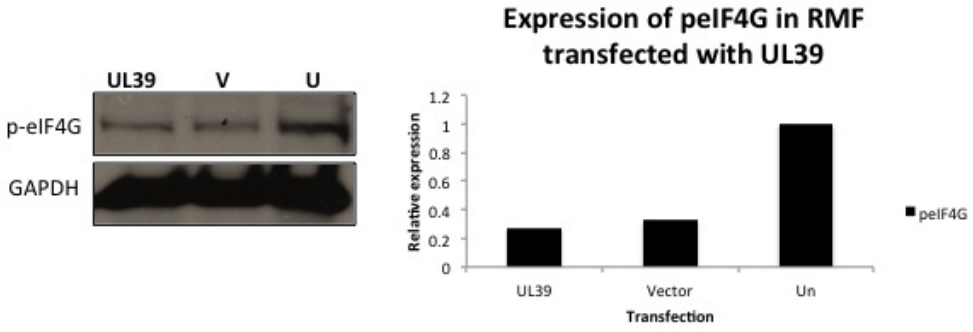

Figure 23: Expression of peIF4G in UL39 transfected RMF

Figure 23: (Left) Western blot analysis of phosphorylated eIF4G in RMF transfected with 2 ug of UL39 pcDNA, empty vector, or untransfected. Antibodies phospho-eIF4G were used to detect the presence of the proteins (Right) Densitometric analysis of phospho-eIF4G normalized to the amount of GAPDH in each sample.

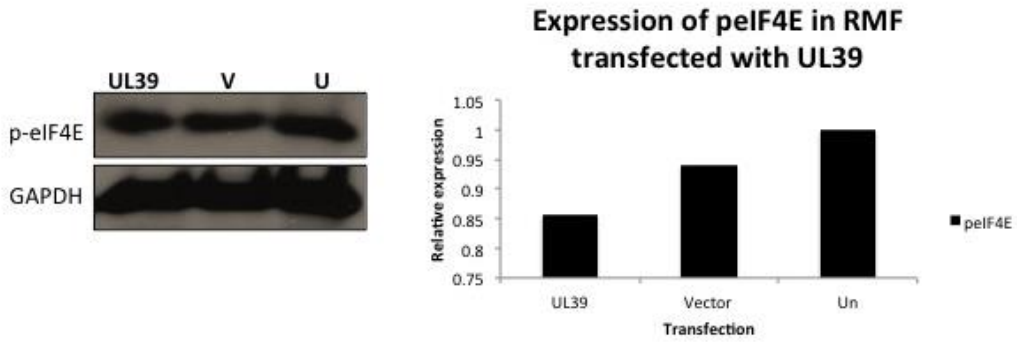

Figure 24: Expression of peIF4E in UL39 transfected RMF

Figure 24: (Left) Western blot analysis of phosphorylated eIF4E in RMF transfected with 2 ug of UL39 pcDNA, empty vector, or untransfected. Antibodies phospho-eIF4E were used to detect the presence of the proteins (Right) Densitometric analysis of phospho-eIF4E normalized to the amount of GAPDH in each sample.

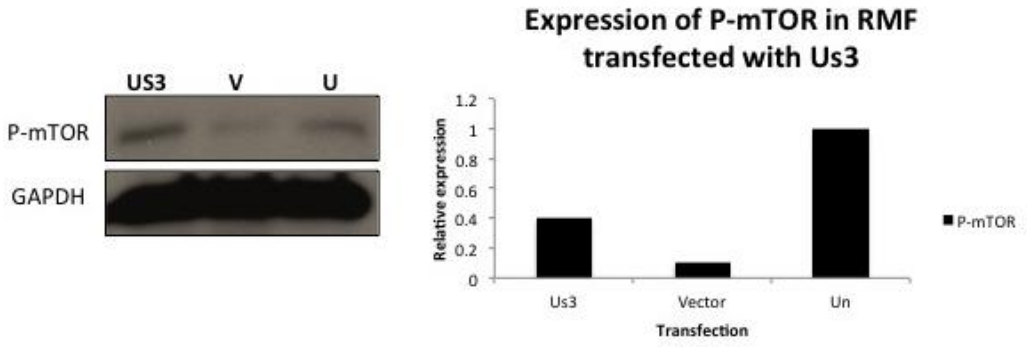

Figure 25: Expression of P-mTOR in US3 transfected RMF

Figure 25: (Left) Western blot analysis of phosphorylated mTOR in RMF transfected with 2 ug of US3 pcDNA, empty vector, or untransfected. Antibodies phospho-mTOR were used to detect the presence of the proteins (Right) Densitometric analysis of phospho-mTOR normalized to the amount of GAPDH in each sample.

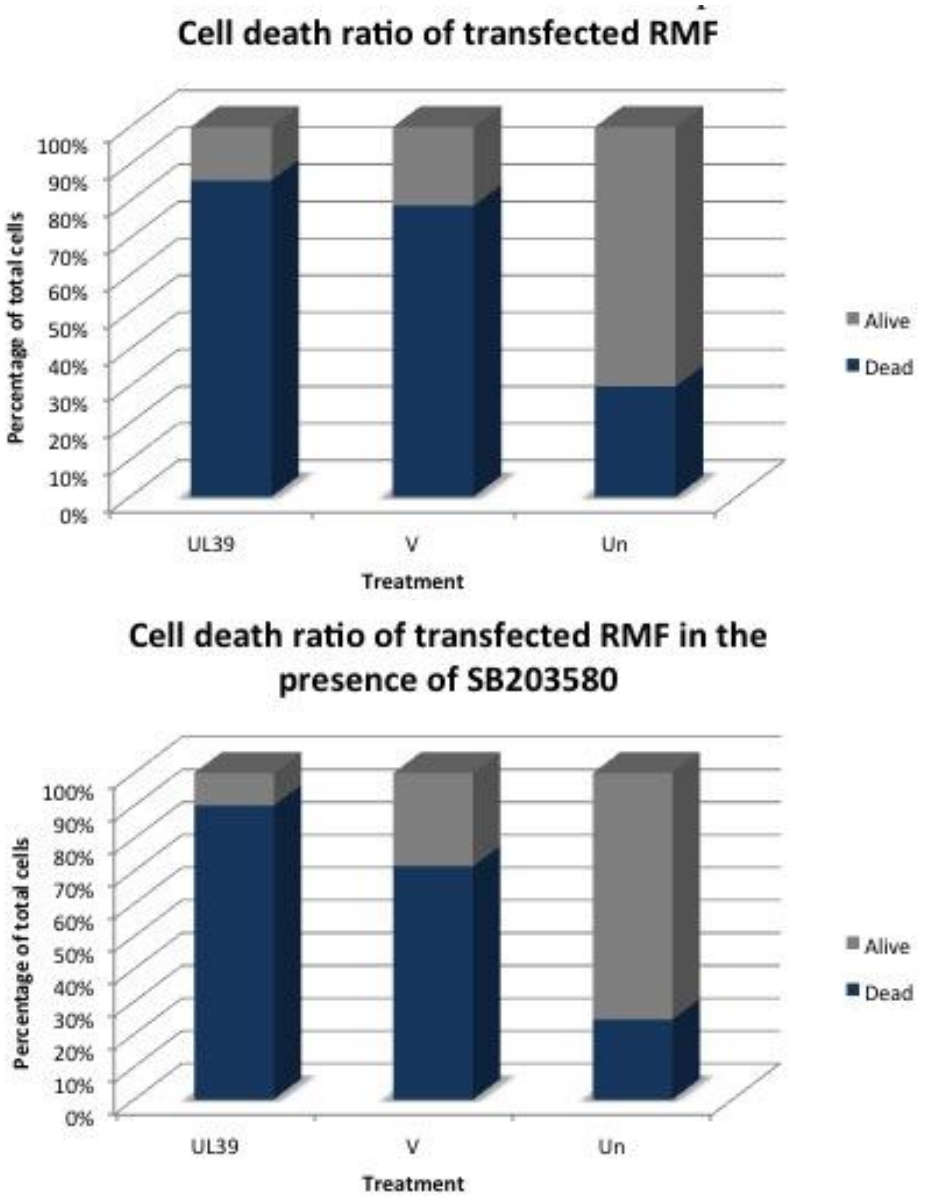

Figure 26: Cell death ratio of UL39 transfected RMF in the presence and absence of SB203580

Figure 26: (Top) Trypan blue exclusion assay of RMF transfected with UL39. Cells were scraped into media and diluted with trypan blue. Dead cells were counted separately from live cells in duplicate. (Bottom) Trypan blue exclusion assay of RMF transfected with UL39 in the presence of SB203580. Cells were scraped into media and diluted with trypan blue. Dead cells were counted separately from live cells in duplicate.

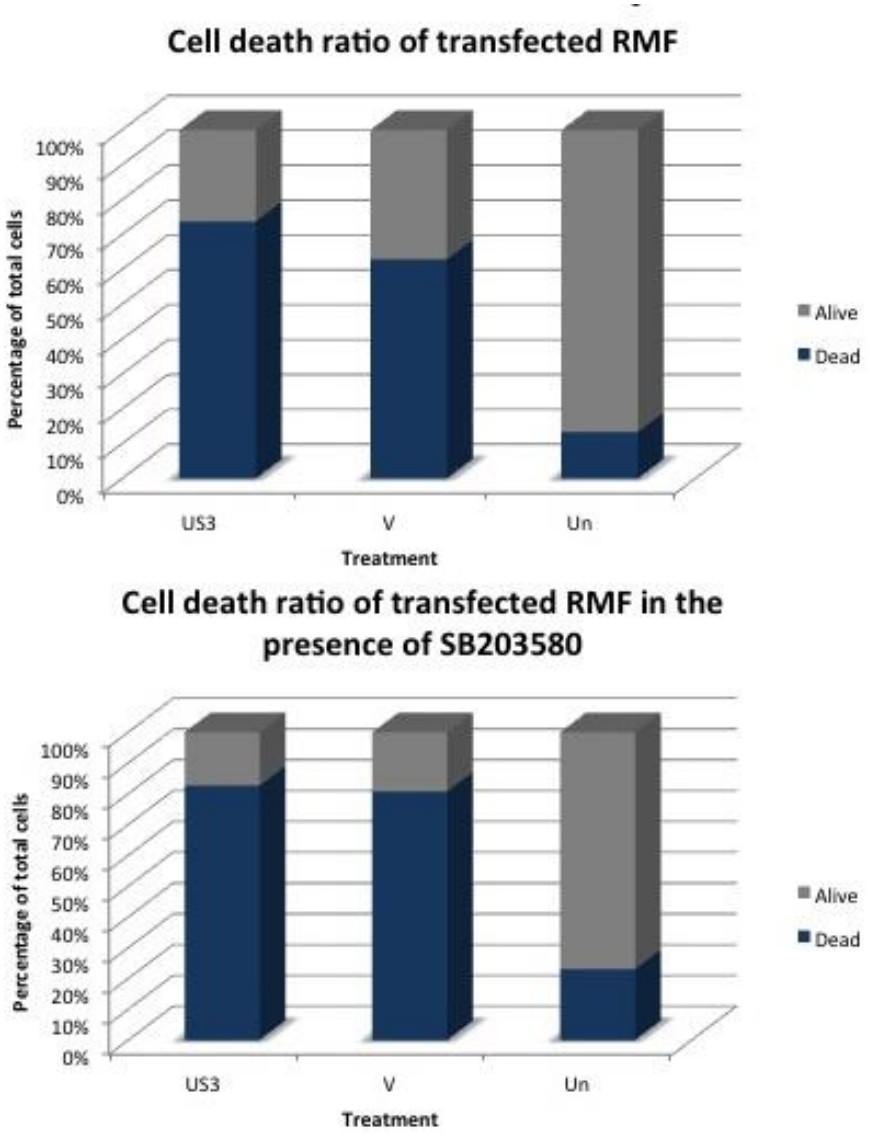

Figure 27: Cell death ratio of US3 transfected RMF in the presence and absence of SB203580

Figure 27: (Top) Trypan blue exclusion assay of RMF transfected with US3. Cells were scraped into media and diluted with trypan blue. Dead cells were counted separately from live cells in duplicate. (Bottom) Trypan blue exclusion assay of RMF transfected with US3 in the presence of SB203580. Cells were scraped into media and diluted with trypan blue. Dead cells were counted separately from live cells in duplicate

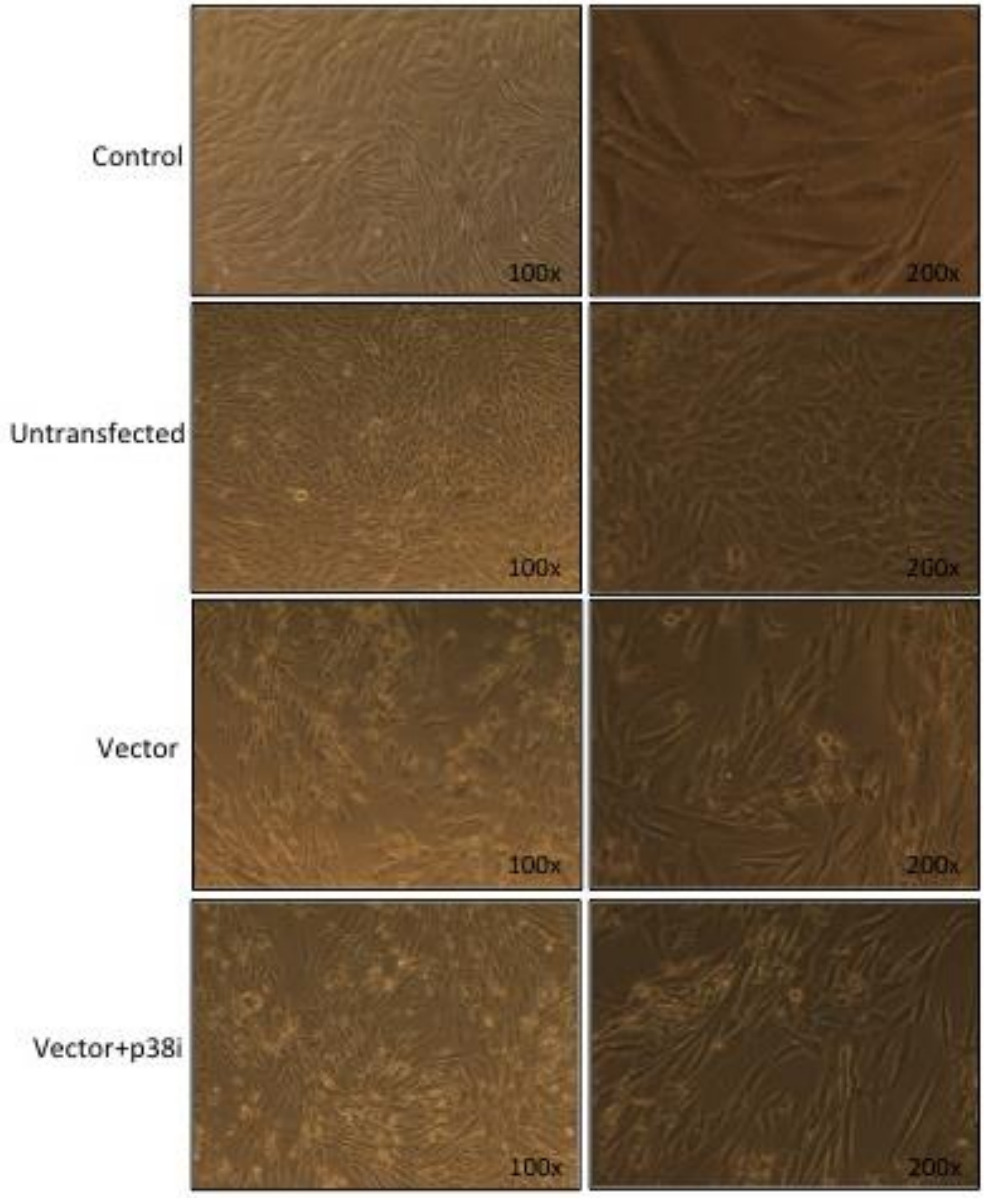

Figure 28: Cytotoxic Effects of transfection in RMF in the presence and absence of SB203580

Figure 28: Control treated RMF and RMF transfected with empty vector in the presence and absence of SB203580.

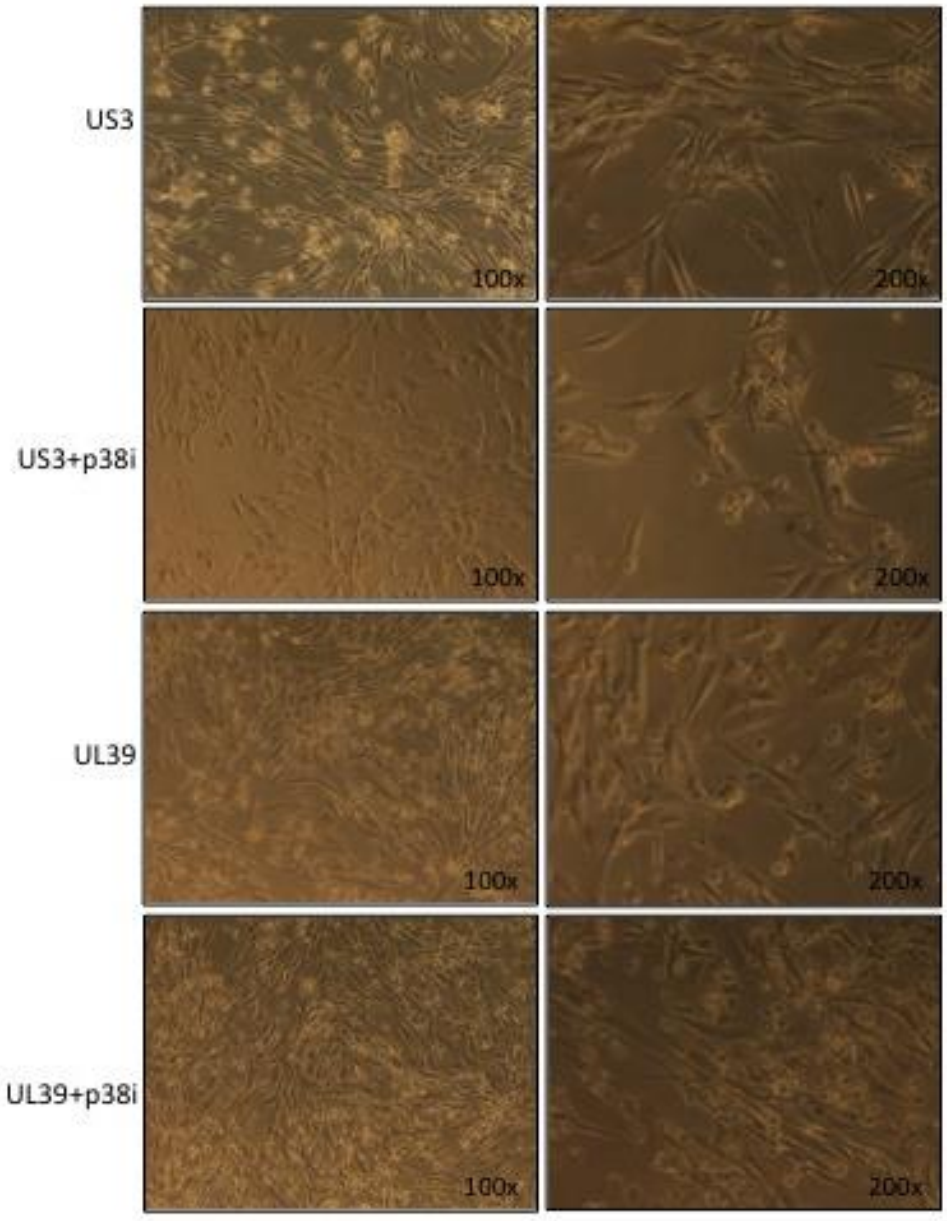

Figure 29: Cytotoxic Effects of transfection in RMF in the presence and absence of SB203580

Figure 29: RMF transfected with US3 (top) or UL39 (bottom) in the presence and absence of SB203580.

4 MATERIALS AND METHODS

4.1 Cells

Rhesus macaque fibroblasts (RMF). Because these cells were not commercially available, they had to be isolated from male and female genital skin. The skin segments were provided by Yerkes Primate Research Center, Emory University, Atlanta Georgia. The tissue was preserved in DMEM upon collection. For isolation, the tissue was removed from the media and prepared as previously described [22]. RMFs were grown in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 18% fetal bovine serum (FBS). These fibroblasts were used to recapitulate the primary site of B virus infection in macaques. Passage numbers 6-10 were used to maintain consistency throughout experiments. For the purposes of this dissertation these cells will be called RMF.

CCD-1079Sk (ATCC® CRL-2097). Human foreskin fibroblasts were purchased from ATCC and grown in minimum essential medium (MEM) supplemented with 10% FBS, 0.1 mM non-essential amino acids, and 1.0 mM sodium pyruvate. These fibroblasts were used to recapitulate the primary site of B virus infection in humans. Passage numbers 6-10 were used to maintain consistency throughout experiments. For the purposes of this dissertation these cells will be called HFF.

Vero (ATCC® CL81). Vero (African green monkey kidney cells) were purchased from ATCC. They were grown in DMEM supplemented with 10% FBS. These cells were used to prepare infected cell lysate for virus stock as well as uninfected cell lysate. They were also used for plaque assay analyses.

CHO-K1 (ATCC® CCL-61). Chinese hamster ovarian (CHO) cells were purchased from ATCC. They were grown in F12K medium supplemented with 10% FBS and 1% antibiotic. These cells were used in B virus protein transfection assays.

Subculture. Prior to passaging, cells were washed twice with phosphate buffered saline (1X PBS). Trypsin-EDTA was added and incubated at 37°C in 5% CO_2 for a period of 3-5 minutes to allow time for cells to detach from the surface of the well or flask. The trypsin was neutralized by adding equal amounts or more of medium containing FBS. The cells were resuspended and placed into new vessels. For all experiments involving plating of cells into wells, trypan blue was added to aliquots of cell suspension for viable cell counting and then 10 µl of the cell solution was loaded onto both sides of a hemocytometer. Plates were seeded equally and grown to sub-confluency. If remaining cells were to be stored for later use, the cell solution was then transferred to a sterile 15 ml conical tube and centrifuged at 1200 rpm for 5 minutes. The supernatants were discarded and the pellets were resuspended in freezing medium (DMEM with 10% DMSO (dimethyl sulfoxide) and 20% FBS). Cells were then aliquotted into sterile cryovials to be frozen initially at -80°C in a designated cell-freezing container. Once frozen, cells were transferred to liquid nitrogen for long-term storage.

4.2 Chemicals

The p38 inhibitor InSolution™ SB203580 (EMD Millipore, cat # 559398) was used to reduce phosphorylation of p38. 100X stocks were prepared in order to use the recommended concentration for IC_{50} of 600 nM in primary cells. This drug competitively binds to the ATP-binding pocket to block phosphorylation of p38α and p38β, but not p38γ or p38δ [53].

The inhibition via SB203580 relies on interaction with the amino acids near or inside of the ATP-binding pocket, specifically with Thr^{106} of p38α and p38β [53, 54]. The inhibitor and stocks were stored at -20°C.

4.3 Viruses

B virus laboratory strain E2490, passage number 74, was propagated in Vero cells and stock was prepared by lysing infected cells through 1-2 rounds of rapid freeze/thaws followed by storage in -80°C. All infections with virus were performed under Bio Safety Level (BSL) – 4 conditions in a registered facility in accordance with the Biosafety in Microbiological and Biomedical Laboratories (BMBL) manual, 5th Edition.

For the purpose of infections, HFF and RMF were counted and seeded into 24 well plates at a desired density that would allow them to reach 90% confluency in 2-3 days. The multiplicity of infection (MOI) used was MOI 5. Mock cell lysate (MCL) was prepared with lysed Vero cells and was used as a control treatment to represent the environment the cells were exposed to during infection, but without the presence of virus. MCL was collected at the same time and in the same way as B virus stock. Virus and MCL were diluted in Hank's Buffered Salt Solution (HBSS) for the appropriate MOI.

For kinetic experiments, media was removed and kept in sterile 50 ml conical tubes for the respective cell lines and will be referred to as 'spent media'. Cells were then washed with 1X PBS twice and then 200 µl of virus solution was added to each well. The plates were then placed in the incubator at 37°C with 5% CO_2 for 1 hour, known as the absorption period. After the absorption period, virus was removed and replaced with 500 µl of spent media for the respective cell type. Samples were collected at various times through 24 hours post infection. MCL solution was prepared to the same MOI and administered to cells in the same manner.

For inhibition experiments, much of the same procedure is followed with the addition of serum starvation 12-18 hours prior to infection. Cells were pre-treated with SB203580 for 1 hour diluted in either MEM or DMEM, depending on cell type on the day of infection. Before infection, the pre-treatment was removed and the cells were washed twice with 1X PBS. The virus solution was prepared with the specified amount of SB203580 and 200 µl of virus solution in the presence of SB203580 was applied to designated wells. The plates were then incubated in 37°C in 5% CO_2 for the absorption period. Virus or MCL was removed and replaced with maintenance medium (MEM/DMEM, 2%FBS, 1% antibiotic) either with or without SB203580. The plates were placed back in incubation for 24 hours, after which the samples were collected.

4.4 SDS-PAGE and Western blot

After infection or MCL treatment, total cell lysates were collected using 75 µl of Laemmli Buffer with protease/phosphatase inhibitor per well. The samples were then stored at -20°C. Each sample was collected in duplicate. Acrylamide gels containing 10% SDS were prepared to analyze protein phosphorylation and expression. The samples were heated at 99°C for 5 minutes to denature the proteins and then immediately placed on ice until they reached room temperature. Samples were loaded into the stacking gel portion of the gel at 15-20 µl of sample per well. The samples were fractionated using electrophoresis for approximately 2 hours at 91V. Proteins present within the gel were then transferred to nitrocellulose membranes for approximately 2 hours at 71V, or overnight at 22V. The membranes were stained with Ponceau S stain to ensure a successful transfer took place. The stain was washed off with 0.1M NaOH for 30 seconds, followed by washes with water two times for 5 minutes each. Blotto (5% non-fat dry milk, 1X TBS, 0.1% Tween 20) was applied to the membranes and they were incubated at room temperature on a rocker for 1 hour. When using one membrane to look at multiple proteins,

sometimes the membranes were cut based on the molecular weights of the proteins to maximize the use of the samples. A dual color ladder marker was used to delineate where the cuts should be made and this was done prior to washing off the Ponceau S stain. After the 1 hour blocking period, the blotto was washed from the membranes using Tris-buffered Saline with Tween-20 (TBS-T) for 10 minutes three times. After each wash, the TBS-T was discarded and fresh TBS-T was used for the next wash. After the three washes the primary antibodies were prepared in primary antibody buffer (5% w/v BSA, 1XTBS, 0.1% Tween®20) to detect phospho-p38 (Cell Signaling Technology, cat #4511), phospho-4EBP-1 (Ser65) (Cell Signaling Technology, cat # 9456), phospho-p44/42 MAPK (ERK1/2) (Cell Signaling Technology, cat #4370) and phospho-eIF4E (Ser209) (Abcam, cat # ab76256) and incubated in 4°C overnight. The following day, the primary antibodies were removed and stored in 4°C if used once and discarded if used twice. The membranes were washed three times for 10 minutes with TBS-T, with new TBS-T used for each wash. Anti-rabbit IgG secondary antibody was prepared according to the manufacturer's instructions and applied to the membranes, which then incubated for 1 hour at room temperature. At the end of 1 hour, the secondary antibody was removed and again membranes were washed three times for 10 minutes with new TBS-T for each wash. At the end of the third wash, membranes were placed in fresh TBS-T in their containers and taken to the core facility provided by the Biology department of Georgia State University, Atlanta Georgia and proteins were imaged by the LAS4000 mini by GE Healthcare. The membranes were blotted to removed excess TBST-T and then placed in a clear plastic sheet. Enhanced Chemiluminescence (ECL) solution from GE Healthcare was prepared according to the manufacturer's instruction and applied, dropwise to the membranes at 1 ml per membrane. A clear plastic cover was applied over the membrane and air bubbles under the cover were eliminated and the membrane was

placed in the machine. The exposure process was done in increments of 1 minute for 5 minutes and the best exposure that was clear but not over developed was chosen for further analysis. ImageQuant software was used to process the image to determine band densities for quantitative analysis. Experimental sample values were then normalized to control samples. Statistics were done using Prism6 by GraphPad Software, Inc.

4.5 Plaque Assay

After infections in the inhibition assays, samples were collected by scraping the bottom of the wells into the medium and collecting the solution into a 1.5 micro-centrifuge tube with an O-ring closure cap. Each sample was collected in duplicate. These samples were stored at -80°C for future use. Veros were seeded into 24 well plates at a desired density to reach approximately 90% confluency in 2-3 days. On the day of infection, serial dilutions (10^{-1} to 10^{-4}) of previously collected samples were made in 1 ml of HBSS. The 10^{-1} dilution was discarded. For infection 200 μl of the serial dilutions were applied to each well in duplicates. The plates were incubated at 37°C in 5% CO_2 for an absorption period. Unabsorbed virus was removed and 1% methylcellulose supplemented with 2% FBS was applied to each well. The plates were placed in the incubator for 48 hours. The methylcellulose was removed and the wells washed twice with 1X PBS to remove residual methylcellulose. The cells were fixed with 100% methanol for 15 minutes. Once the plates were dried, the wells were stained with crystal violet and plaques were observed under microscope and counted to determine plaque forming units (PFU).

4.6 Transfection Assays

pcDNA vectors from Invitrogen® were excised and B virus genes UL39 and US3 were each inserted into the sequence. These plasmids were a gracious gift from Dr. Irina Patrusheva. CHO cells were seeded into two 24 well plates and grown to approximately 70% confluency.

Culture medium was discarded and the cells were washed one time with 1X PBS. Transfection medium consisting of Opti-MEM® with 1% FBS was added to the wells. Lipofectamine 3000® reagents were used according to the manufacturer's protocol [78]. Initially two micrograms and four micrograms of plasmid DNA were transfected into cells to optimize the amount of DNA needed for subsequent experiments. After 48 hours, the medium was discarded and the cells were gently washed with 1X PBS. Laemmli buffer was used to collect whole cell lysate for analysis.

For the transfection and simultaneous inhibition assay, the same protocol was followed for transfection with a pre-treatment of cells for 1 hour with SB203580 prior to the transfection. In transfection medium, SB203580 was added at an IC_{50} of 600 nM. The cells were collected after 24 hours with Laemmli buffer.

For transfection of RMF, the times post transfection were extended to 48 hours in the case of UL39 transfected cells and 72 hours in the case of US3 transfected cells.

4.7 Trypan Blue Exclusion Assay

RMF were transfected according to transfection assay indicated above. On the days of collection, cells were imaged microscopically and then scraped with a pipette tip into the medium. Thirty microliters of cell solution was transferred to an Eppendorf tube with 30 ul of trypan blue. Ten microliters were loaded onto both sides of a hematocytometer and the number of dead cells (stained blue due disruption of membrane integrity) and the number of live cells (unstained due to intact membrane) were counted separately in duplicate for each sample.

5 CONCLUSIONS

5.1 Understanding zoonotic viruses: what we know about their behaviors in natural vs. foreign hosts

New infectious diseases are emerging every year and have an immense impact on human health [80]. Most infectious diseases are caused by viruses, the majority of which originated in an animal species including Ebola, HIV, hantaviruses, Hendra and Nipah viruses, severe acute respiratory syndrome (SARS), coronavirus, and influenza A viruses [81, 82]. Transmission of these zoonotic viruses varies and with the growth in size and mobility of human populations, environmental changes, and spread of agricultural practices that promote more contact with animals, the frequency of exposure to these pathogens and dissemination has greatly increased [83]. Tools developed to quickly detect these viruses have been improved in recent years leading to more rapid detection and diagnosis in humans as well as rapid identification of reservoir hosts [84, 85]. The introduction and spread of these pathogens in foreign host populations is a popular topic of research [86, 87], but host-dependent responses triggered by host-virus interactions are still poorly understood. These host-virus interactions are critical in determining the outcome of infection and the likelihood of transmission.

In the natural hosts, these viruses have little to no effect on the individual animal or the species population as a whole due in part to the theory that these viruses have co-evolved with their respective hosts for thousands of years and have developed a tolerant synergy with one another. The co-evolutionary relationship between a virus and its natural host is such that the body of the natural host responds to the presence of the virus with little to no significant physical symptoms and the virus is permitted to replicate within the host cell and be disseminated through the route of transmission used with little to no issue. In foreign hosts, infection with these viruses

can result in fatal disease while causing only mild infections in their natural hosts. Different zoonotic viruses affecting humans can be characterized by unique biological and clinical manifestations. These manifestations in humans are complex and poorly understood. The first step in establishment of a new host species for a virus is the manipulation of intercellular interactions between the virus and the host. Modification of various signaling pathways within the infected cell of the new host is crucial for establishing the tolerant synergy a virus has with its natural host, therefore signaling pathways are one of the first targets of virus control and manipulation. Determining what, if any, effectors may impact viral replication kinetics within the natural vs. foreign hosts may provide valuable information about why some viruses cause severe disease in humans. Assessing possible effectors, such as stress response pathways, has become a hot topic of research in regards to determining the difference in outcomes of infection in zoonotic viruses.

5.2 The role of MAPK p38 stress response pathway in viral infection of susceptible hosts

The MAPK signaling cascades are involved in many cellular activities such as gene expression, cell-cycle regulation, metabolism, motility, survival, and apoptosis. Among the MAPKs, the ERKs, JNKs, and p38 MAPKs are the most studied [88, 89]. Canonically, ERKs are usually activated in response to mitogens like growth factors and phorbol esters, and the JNKs and p38 MAPKs are responsive to stressful stimuli like osmotic shock, cytokine stimulation, and infection [90]. The p38 MAPK is strongly activated by various physical and chemical stresses like oxidative stress, ultraviolet irradiation, hypoxia, ischemia and cytokines. The p38 stress response plays a role in apoptosis, differentiation, proliferation and inflammation and there is a substantial amount of evidence indicating that p38 activity is critical for normal immune function. The p38 MAPK pathway has been implicated as having a role in multiple pathologies

such as inflammation, cancer and virus infection and is highly conserved between species. Cytokine production has been shown to be mediated by p38α in mice [91]. Pro-inflammatory cytokine (PIC) production is a major stimulant of chronic inflammatory and rheumatic diseases such as rheumatoid arthritis and Crohn's disease and there is evidence that PIC also plays a role in heart failure and ischaemic retinopathies [92, 93]. Blocking the production of PIC has become an attractive therapeutic strategy and a number of pharmaceutical companies have been developing drugs targeting p38α to block PIC production, some of which have advanced to human clinical trials such as AMG 548 (Amgen) and SCIO 323(Scios, Johnson and Johnson) [94]. Recently it has been shown that inhibition of p38 impairs influenza virus-induced host responses in mice. Avian influenza viruses can be highly pathogenic and induce severe inflammatory reactions in birds and humans. One characteristic of these viruses is the induction of cytokines that enhance viral pathogenesis. This enhancement appears to involve the hyperactivation of p38 during infection. Influenza A virus activates all four of the know MAPK cascades. It was observed that p38 has an important role in highly pathogenic avian influenza virus (HPAIV)-induced cytokine and chemokine dysregulation, therefore making it a new possible target for antiviral therapy for influenza viruses. This study also implicated a role for p38 in recruitment of immune cells during an influenza infection by regulating chemotactic cytokines such as CXCL9, -10, -11, and CCL5 in endothelial cells [95]. The role of p38 in viral infection and pathogenesis is still being investigated and its role may differ depending on the type of virus and the infected host, but it is clear that the p38 stress response plays a pivotal role in host-virus interactions. It is for this reason that we investigated the effect of p38 on B virus infection in the natural and foreign hosts.

We selected the MAPK p38 stress pathway to study its function during zoonotic viral invasion, using a cell-based model of species-specific infection. In the absence of adequate animal models to study zoonotic infection and the costly *in vivo* studies in the natural host of B virus, we established a cell model system to investigate potential differences that may exist in the natural versus the human host cells targeted upon virus entry. The use of this cell model system allows the use of many essential controls and the isolation of specific innate defense pathways. The particular interest for this dissertation research was to enhance our understanding of cell translational machinery and how the use of such machinery may differ between hosts and in the presence or absence of virus infection, thus we focused our studies on the translation factor eIF4E, which is crucial to cap-dependent translation, and additionally on 4E-BP1, one of the critical regulators of eIF4E in addition to p38 and ERK. To better understand the use of translation machinery by the two cell species chosen for this research in the context of virus infection, we must first understand the co-evolution of B virus with macaques and other herpesviruses with their respective hosts.

5.3 The co-evolution of herpesviruses and their hosts

The first step in understanding why a virus is relatively harmless in its natural host, but devastating in a foreign host is to first understand what is occurring in the natural host. Herpesviruses belonging to the genus *Simplex* are, for the most part, species-specific. Almost every species of animal has a herpesvirus that has co-evolved with it and the herpesvirus is specific for that particular species. This trait of species restriction that is a characteristic of most simplex viruses is a product of virus-host interactions being selected for to allow peaceful co-existence. As with most things, there are exceptions to every rule. Herpes B virus, though endemic in macaque monkeys, is capable of infecting human hosts. Currently there is only one

reported case of human to human spread of B virus, indicating that the virus is less efficient at transmission within its human host. The human herpes simplex viruses type 1 and 2 may at one point been zoonotic infections that were able to take hold within the human population and establish a co-evolutionary relationship, though which human herpes virus infected humans first is still a topic of debate. Many viruses that humans are familiar with today such as the influenza viruses and the human immunodeficiency virus (HIV) are products of zoonotic viruses that successfully jumped the species barrier and are just as effective if not more so in their human hosts. The first step in jumping the species barrier is to alter these virus-host interactions to suit the virus within its new host. From there, the virus can start to spread itself first among a small population of its new host and eventually spread to larger populations and either reach an epidemic status or become endemic wherein the virus will evolve to co-exist within that new host population. Regardless of the severity of infection from a zoonotic host and the amount of time it takes to settle within a new host population, it always begins with altering the interactions within the cell to the virus.

5.4 Regulation of MAPK p38 pathway during B virus infection

The first sight of infection for B virus, before the virus enters the sensory neurons, is the skin. The skin is an essential barrier to infection and it contains many layers, one of which is the dermis that houses fibroblast cells. Fibroblasts are important contributors to innate immunity functions in the skin and are capable of secreting a variety of cytokines, chemokines, and antimicrobial peptides. With our cell culture model that represents the cells that would first become infected at a primary site of infection in both macaques and humans, we can investigate the initial events following B virus infection and how these events affect downstream factors. The p38 MAPK pathway, which leads to cytokine secretion and apoptosis is a crucial event that

is triggered by infection with B virus. The natural and foreign host cell model has allowed us to analyze this pathway in both cell types infected with B virus and observe any host-dependent events that may occur. Of the four known MAPKs, p38 and JNK are triggered by stressful stimuli as opposed to the others that are stimulated by non-threatening mitogens. During B virus infection and other virus infections, p38 is activated presumably to trigger a cytokine and cellular defense response. Though other MAPKs such as the ERKs may initially be activated, ERK activity is eventually downregulated by viruses. Though the activation of p38 is undoubtedly to help the host, the activation of this pathway can also be harmful if not regulated properly as is evidenced in the case of avian influenza studies [95]. The overstimulation of this pathway can lead to prolonged pro-inflammatory cytokines, which can also be harmful to the hosts in addition to the infection the cells are attempting to fight. Our studies indicate that there may be a host-dependent regulation of the p38 MAPK stress response pathway in regards to B virus infection.

In the natural host, p38 activity is increased during infection and translation is initiated. This indicates that the cells are activating the pathway to fight the infection. The increase in activity is most apparent at 24 hours post infection, which is after the virus has completed at least one round of successful replication. Activation of translation initiation factor eIF4E is also observed, though a significant increase does not occur. Our data suggest that although B virus infection of human cells targeted at virus entry does not significantly impact eIF4E phosphorylation to enhance or decrease mRNA translation via the MAPK p38 pathway, macaque cells may effectively use this pathway to limit virus replication to levels that can be controlled by host defenses. This indicates that, although the cell is infected with a virus, the cell is tolerating the infection to an extent. There appears to be a balance struck between the natural host cell and the virus to protect the host, yet still allows successful virus replication. This is the first evidence

of host-dependent response to B virus infection in the context of cellular translation indicating that if a divergence between hosts was in affect during infection, it involves manipulation of cellular translation machinery. Cellular translation at this point may be utilized by the cell to translate proteins to aid in cellular defense or by the virus to produce viral proteins.

Because 4E-BP1 hyperphosphorylation was identified as a critical mechanism which HSV1 and human hosts selected during their co-evolution, we asked whether this particular aspect of infection was conserved between B virus and its natural host, macaques. When p38 activity is inhibited, this leads to a decrease in inactive 4E-BP1, which should also lead to a decrease in eIF4E activation and translation, but does not implicating a role of p38 in regulation of 4E-BP1 phosphorylation. This observation suggested that there should be more hypophosphorylated 4E-BP1, but when expression levels were analyzed it was observed that there was no change in hypophosphorylated 4E-BP1 when p38 activity was inhibited. To determine what caused the decrease in hyperphosphorylated 4E-BP1, we analyzed the expression pattern of the positive regulator, PPM1G, and the negative regulator, mTOR, of 4E-BP1 and discovered that neither were significantly upregulated or down-regulated in the absence of p38; however, a recent publication has determined that the presence of p38 can enhance activation of downstream effectors of mTOR. Together, these data suggest that although p38 does not directly cause the decrease in hyperphosphorylated 4E-BP1, in the absence of p38 mTOR is not stimulated to phosphorylate 4E-BP1. The finding that 4E-BP1 hyperphosphorylation and inactivation is modulated by B virus infection in both hosts, but is only regulated by the presence of p38 phosphorylation in macaques further indicates that the manipulation of the p38 pathway and cellular translation is being targeted by B virus in an effort to establish successful infection of the new host. It was further determined that although p38 may play a role in eIF4E

phosphorylation during infection, it is not necessary for eIF4E activation. This was evidenced by our investigation of the effect of p38 on virus titers in the natural and foreign hosts.

We observed a significant increase in B virus titers despite a decrease in inactive 4E-BP1. After further investigation it was determined that there was not more active 4E-BP1 in inhibited cells, but rather that there was less phosphorylation of 4E-BP1 occurring due to the indirect regulation of mTOR by amino acids that require basal activity levels of p38 [77]. Our data suggests that p38 plays a regulatory role in B virus replication in the natural host. In the absence of p38 activity, there is almost a log increase in virus titers.

MAPK p38 appears to play a pivotal role in the balance between natural host survival and B virus infection, but has not adapted the same role in the foreign host. In human fibroblasts, little is changed when p38 activity is inhibited. Activation of eIF4E still occurs, but is not significantly upregulated or downregulated as is the case with macaque fibroblasts as well. Activation of eIF4E is known to be triggered by other proteins such as ERK, the MAPK responsive to non-stressful events; however, ERK was shown to be downregulated during B virus infection in both hosts indicating that it plays no role in the activation of eIF4E during B virus infection. Still others have hypothesized that protein kinase C (PKC) is capable of phosphorylated eIF4E directly *in vitro*, but the mechanism of action *in vivo* is not known [96]; however, this occurrence has only been observed once to date and has been disputed by several other sources. It is possible though that PKC along with PKA may stimulate eIF4E phosphorylation indirectly by activating pathways that phosphorylate MNK1, which in turn phosphorylates eIF4E [97, 98]. Hyperphosphorylation and inactivation of 4E-BP1 in human fibroblasts is increased in response to B virus infection as is observed in macaques, but is not decreased when p38 activity is inhibited indicating that in humans p38 does not have a

regulatory effect on 4E-BP1 in the context of B virus infection. When p38 activity is inhibited viral titers remain the same as seen in B virus infected cells when p38 is active. This implies that p38 plays a role in virus regulation in the natural host but not a significant role in the foreign host. Two possibilities surface when we consider this. First, that the virus may not be able to manipulate p38 in the human host yet or, perhaps more likely, p38 in humans hasn't developed capabilities to manage B virus.

5.5 A novel role for B virus ICP6

Several independent investigators have provided evidence that certain specific viral proteins have the ability to control translation machinery or mimic the machinery. For example, HSV1 viral proteins have been shown to manipulate formation of the eIF4F complex [29], and hantavirus provides a protein that mimics the complex to enhance viral mRNA translation [30]. Therefore it is possible that like HSV1, a B virus protein can interfere with one or both of these processes involving eIF4E.

The effect of viral proteins on cellular translational machinery is well documented. As stated earlier, other viruses such as HSV1 and hantavirus can manipulate or mimic this function to enhance viral mRNA translation. Because p38 plays an important role in regulating viral replication in the natural host, it was important to analyze the effect of viral proteins on macaque fibroblasts in the presence or absence of p38 activity. When cells are transfected with B virus ICP6, it is expected that there will be an increase in eIF4G activity and eIF4E activity as is the case with HSV-1 ICP6. Our data shows that there is no significant upregulation of either and further, that eIF4E and eIF4G regulation is independent of p38 activity both in the presence and absence of ICP6. One possible reason for this unexpected result is that, because CHO cells are not the natural host for this virus, the viral proteins may behave differently in CHO cells than

they behave in the RMFs. The second possibility is that B virus ICP6 function differs from that of its counterpart in HSV1. Despite the fact that HSV1, HSV2, and BV are genetically similar, the protein product of gene UL39 is only 77% similar between B virus and HSV1 and 78% similar between B virus and HSV2. This indicates that the proteins may be different enough from one another between the viruses that they may have different functions altogether. It is possible that although UL39 is confirmed to produce a ribonucleotide reductase in HSV1 and HSV2 and is purported to prodcue a ribonucleotide reductase in B virus, the protein itself may also have different functions in addition to the main function and these secondary functions may differ between viruses. In this case, the identification of a difference in function of ICP6 between HSV1 and BV would be a novel finding in the field of herpesvirus research, as it has been assumed that functions of viral proteins are conserved through the families of herpesviruses.

A side-by-side analysis of the protein product of UL39 between HSV1, HSV2, and B virus is necessary to determine the various functions of this conserved protein and whether these proteins perform different functions dependent upon the virus to which it belongs.

5.6 A novel role for B virus US3

Another candidate viral protein for cellular translational regulation was US3, a kinase encoded by the herpesvirus genome. In US3 transfected cells, we see an upregulation of mTOR as is also seen with HSV1 US3. This is followed by a subsequent increase in hyperphosphorylation of 4E-BP1. This is reflective of what we see in the natural host cells infected with B virus. When p38 activity is ablated, there is a decrease of mTOR activity and hyperphosphorylation of 4E-BP1 decreases when US3 is present, which also correlates with our data in the natural host. The phosphorylation of mTOR is significantly enhanced by the presence of B virus US3. This result reflects our expected findings as US3 has been shown to enhance

mTOR phosphorylation and activity during HSV1 infection. We also showed that this activation depends on the presence of p38 activity. In the absence of p38, mTOR phosphorylation is greatly decreased even in the presence of US3. This indicates that mTOR phosphorylation does require p38 activity to an extent. Lastly, we found that phosphorylation of 4E-BP1 at serine 65 is maintained in the presence of US3; however, this is also dependent on p38 activity. This result was also expected as 4E-BP1 inactivation is directly affected by mTOR activity. Also, previous studies have shown that p38 activity does stimulate certain functions of mTOR, such as 4E-BP1 phosphorylation, but does not affect mTOR phosphorylation itself. The similarity of US3 between B virus and HSV1 and 2 is low, despite the appearance of similar functionality of the protein between viruses. These results in regards to B virus proteins ICP6 and US3 are intriguing and may bring novel findings to the fields of B virus and herpesvirus research, but it is pertinent that the roles of ICP6 and US3 be examined in the natural host cells. Transfection of RMF using lipofection was not achieved due to high cytotoxicity of lipfection reagents. These cells are primary fibroblasts that are considerably older than other primary cell lines that have successfully been transfected using lipofection techniques. Lipofection is currently one of the most popular methods of transfection and has been indicated as the method of choice for difficult cell types such as primary cells; however, our data shows that reagents used in lipofection are significantly harmful to these cells and result in anywhere from 70-95% cell death in cells exposed to the reagents. Viral protein product is detected via the V5 epitope located in the pcDNA plasmids, but the reagents are so cytotoxic that any effect caused by the presence of the viral proteins is masked by the damage done to the cells. Taken all together, this work indicates that p38 does play a substantial role in both the cellular and viral workings within the natural host. This novel identification of the role of p38 during B virus infection in its natural host along

with the data that shows this role is host-dependent will be extremely helpful in identifying why B virus is so deadly in a foreign host but has managed to co-exist relatively peacefully with its natural host.

The findings outlined in this dissertation lay crucial ground work in determining how this zoonotic virus regulates cellular pathways differentially between hosts, which is the first step in successful zoonotic transmission of a virus to a new host. These data demonstrate how a zoonotic virus, in this case B virus, can regulate a cellular process in the natural host with which it has co-evolved, but not in the foreign human host and suggest how these interactions can contribute to the host-specific differences in the outcome of virus infection. Once it is known how a zoonotic virus interacts with its natural host cell and its human host cells, we will then have the possibility of designing intelligent antiviral therapeutics and vaccines.

REFERENCES

1. Gay, F., Holden M., *The herpes encephalitis problem.* Journal of Infectious Diseases, 1933. **53**(3): p. 287-303.

2. Sabin, A., Wright WM, *Acute ascending myelitis following a monkey bite, with the isolation of a virus capable of reproducing the disease.* Journal of Experimental Medicine, 1934. **59**(2): p. 115-136.

3. Eberle, R. and J. Hilliard, *The simian herpesviruses.* Infectious agents and disease, 1995. **4**(2): p. 55-70.

4. Perelygina, L., et al., *Complete sequence and comparative analysis of the genome of herpes B virus (Cercopithecine herpesvirus 1) from a rhesus monkey.* Journal of virology, 2003. **77**(11): p. 6167-77.

5. Ohsawa, K., et al., *Sequence and genetic arrangement of the U(S) region of the monkey B virus (cercopithecine herpesvirus 1) genome and comparison with the U(S) regions of other primate herpesviruses.* Journal of virology, 2002. **76**(3): p. 1516-20.

6. Whitley, R.J. and B. Roizman, *Herpes simplex virus infections.* Lancet, 2001. **357**(9267): p. 1513-8.

7. Sawtell, N.M., *The probability of in vivo reactivation of herpes simplex virus type 1 increases with the number of latently infected neurons in the ganglia.* Journal of virology, 1998. **72**(8): p. 6888-92.

8. Hilliard, J.K., Eberle, R., Lipper, S.L., *Herpesvirus simiae (B Virus): Replication of the Virus and Identification of Viral Polypeptides in Infected Cells.* Archives of Virology, 1987. **93**: p. 185-198.

9. Weigler, B.J., *Biology of B virus in macaque and human hosts: a review.* Clinical infectious diseases : an official publication of the Infectious Diseases Society of America, 1992. **14**(2): p. 555-67.

10. Weigler, B.J., et al., *Epidemiology of cercopithecine herpesvirus 1 (B virus) infection and shedding in a large breeding cohort of rhesus macaques.* The Journal of infectious diseases, 1993. **167**(2): p. 257-63.

11. Keeble, S.A., G.J. Christofinis, and W. Wood, *Natural virus-B infection in rhesus monkeys.* The Journal of pathology and bacteriology, 1958. **76**(1): p. 189-99.

12. Zwartouw, H.T. and E.A. Boulter, *Excretion of B virus in monkeys and evidence of genital infection.* Laboratory animals, 1984. **18**(1): p. 65-70.

13. Lees, D.N., et al., *Herpesvirus simiae (B virus) antibody response and virus shedding in experimental primary infection of cynomolgus monkeys.* Laboratory animal science, 1991. **41**(4): p. 360-4.

14. *B-virus infection in humans--Pensacola, Florida.* MMWR. Morbidity and mortality weekly report, 1987. **36**(19): p. 289-90, 295-6.

15. Davenport, D.S., et al., *Diagnosis and management of human B virus (Herpesvirus simiae) infections in Michigan.* Clinical infectious diseases : an official publication of the Infectious Diseases Society of America, 1994. **19**(1): p. 33-41.

16. Holmes, G.P., et al., *B virus (Herpesvirus simiae) infection in humans: epidemiologic investigation of a cluster.* Annals of internal medicine, 1990. **112**(11): p. 833-9.

17. Sabin, A.B. and A.M. Wright, *Acute Ascending Myelitis Following a Monkey Bite, with the Isolation of a Virus Capable of Reproducing the Disease.* The Journal of experimental medicine, 1934. **59**(2): p. 115-36.

18. Huff, J.L. and P.A. Barry, *B-virus (Cercopithecine herpesvirus 1) infection in humans and macaques: potential for zoonotic disease.* Emerging infectious diseases, 2003. **9**(2): p. 246-50.

19. Tischer, B.K. and N. Osterrieder, *Herpesviruses--a zoonotic threat?* Veterinary microbiology, 2010. **140**(3-4): p. 266-70.

20. Parrish, C.R., et al., *Cross-species virus transmission and the emergence of new epidemic diseases.* Microbiology and molecular biology reviews : MMBR, 2008. **72**(3): p. 457-70.

21. Desrosiers, R.C., *The value of specific pathogen-free rhesus monkey breeding colonies for AIDS research.* AIDS research and human retroviruses, 1997. **13**(1): p. 5-6.

22. Farah-Abraham, R.M., *B Virus Infection Activates p38 and JNK Pathways Differentially in Cells From Macaque Versus Human Hosts: Exploring Inflammation and Apoptosis*, in *Biology Dissertations*. 2011, Georgia State University: Atlanta.

23. Cuandro, A., Angel R. Nebreda, *Mechanisms and functions of p38 MAPK signalling.* Biochemical Journal, 2010(429): p. 403-417.

24. Zhang, Y.L. and C. Dong, *MAP kinases in immune responses.* Cellular & molecular immunology, 2005. **2**(1): p. 20-7.

25. Han, J., et al., *A MAP kinase targeted by endotoxin and hyperosmolarity in mammalian cells.* Science, 1994. **265**(5173): p. 808-11.

26. Jiang, Y., et al., *Characterization of the structure and function of a new mitogen-activated protein kinase (p38beta).* The Journal of biological chemistry, 1996. **271**(30): p. 17920-6.

27. Lechner, C., et al., *ERK6, a mitogen-activated protein kinase involved in C2C12 myoblast differentiation.* Proceedings of the National Academy of Sciences of the United States of America, 1996. **93**(9): p. 4355-9.

28. Jiang, Y., et al., *Characterization of the structure and function of the fourth member of p38 group mitogen-activated protein kinases, p38delta.* The Journal of biological chemistry, 1997. **272**(48): p. 30122-8.

29. Sabio, G., et al., *p38gamma regulates the localisation of SAP97 in the cytoskeleton by modulating its interaction with GKAP.* The EMBO journal, 2005. **24**(6): p. 1134-45.

30. Ashwell, J.D., *The many paths to p38 mitogen-activated protein kinase activation in the immune system.* Nature reviews. Immunology, 2006. **6**(7): p. 532-40.

31. Enslen, H., J. Raingeaud, and R.J. Davis, *Selective activation of p38 mitogen-activated protein (MAP) kinase isoforms by the MAP kinase kinases MKK3 and MKK6.* The Journal of biological chemistry, 1998. **273**(3): p. 1741-8.

32. Kyriakis, J.M. and J. Avruch, *Mammalian mitogen-activated protein kinase signal transduction pathways activated by stress and inflammation.* Physiological reviews, 2001. **81**(2): p. 807-69.

33. Marasa, B.S., et al., *Increased MKK4 abundance with replicative senescence is linked to the joint reduction of multiple microRNAs.* Science signaling, 2009. **2**(94): p. ra69.

34. Mace, G., et al., *Phosphorylation of EEA1 by p38 MAP kinase regulates mu opioid receptor endocytosis.* The EMBO journal, 2005. **24**(18): p. 3235-46.

35. Lee, J.C., et al., *A protein kinase involved in the regulation of inflammatory cytokine biosynthesis.* Nature, 1994. **372**(6508): p. 739-46.

36. Kontoyiannis, D., et al., *Impaired on/off regulation of TNF biosynthesis in mice lacking TNF AU-rich elements: implications for joint and gut-associated immunopathologies.* Immunity, 1999. **10**(3): p. 387-98.

37. Arthur, J.S., *MSK activation and physiological roles.* Frontiers in bioscience : a journal and virtual library, 2008. **13**: p. 5866-79.

38. Mahalingam, M. and J.A. Cooper, *Phosphorylation of mammalian eIF4E by Mnk1 and Mnk2: tantalizing prospects for a role in translation.* Progress in molecular and subcellular biology, 2001. **27**: p. 132-42.

39. Fukunaga, R. and T. Hunter, *MNK1, a new MAP kinase-activated protein kinase, isolated by a novel expression screening method for identifying protein kinase substrates.* The EMBO journal, 1997. **16**(8): p. 1921-33.

40. Waskiewicz, A.J., et al., *Mitogen-activated protein kinases activate the serine/threonine kinases Mnk1 and Mnk2.* The EMBO journal, 1997. **16**(8): p. 1909-20.

41. Scheper, G.C., et al., *The N and C termini of the splice variants of the human mitogen-activated protein kinase-interacting kinase Mnk2 determine activity and localization.* Molecular and cellular biology, 2003. **23**(16): p. 5692-705.

42. Ueda, T., et al., *Mnk2 and Mnk1 are essential for constitutive and inducible phosphorylation of eukaryotic initiation factor 4E but not for cell growth or development.* Molecular and cellular biology, 2004. **24**(15): p. 6539-49.

43. Gingras, A.C., B. Raught, and N. Sonenberg, *eIF4 initiation factors: effectors of mRNA recruitment to ribosomes and regulators of translation.* Annual review of biochemistry, 1999. **68**: p. 913-63.

44. Gingras, A.C., B. Raught, and N. Sonenberg, *Regulation of translation initiation by FRAP/mTOR.* Genes & development, 2001. **15**(7): p. 807-26.

45. Walsh, D., M.B. Mathews, and I. Mohr, *Tinkering with translation: protein synthesis in virus-infected cells.* Cold Spring Harbor perspectives in biology, 2013. **5**(1): p. a012351.

46. Dobrikova, E., et al., *Herpes simplex virus proteins ICP27 and UL47 associate with polyadenylate-binding protein and control its subcellular distribution.* Journal of virology, 2010. **84**(1): p. 270-9.

47. Parrish, S. and B. Moss, *Characterization of a second vaccinia virus mRNA-decapping enzyme conserved in poxviruses.* Journal of virology, 2007. **81**(23): p. 12973-8.

48. Feng, P., D.N. Everly, Jr., and G.S. Read, *mRNA decay during herpes simplex virus (HSV) infections: protein-protein interactions involving the HSV virion host shutoff protein and translation factors eIF4H and eIF4A.* Journal of virology, 2005. **79**(15): p. 9651-64.

49. Sandri-Goldin, R.M., *The many roles of the highly interactive HSV protein ICP27, a key regulator of infection.* Future microbiology, 2011. **6**(11): p. 1261-77.

50. Walsh, D. and I. Mohr, *Phosphorylation of eIF4E by Mnk-1 enhances HSV-1 translation and replication in quiescent cells.* Genes & development, 2004. **18**(6): p. 660-72.

51. Hargett, D., T. McLean, and S.L. Bachenheimer, *Herpes simplex virus ICP27 activation of stress kinases JNK and p38.* Journal of virology, 2005. **79**(13): p. 8348-60.

52. Hernandez, G. and P. Vazquez-Pianzola, *Functional diversity of the eukaryotic translation initiation factors belonging to eIF4 families.* Mechanisms of Development, 2005. **122**(7-8): p. 865-76.

53. Young, P.R., et al., *Pyridinyl imidazole inhibitors of p38 mitogen-activated protein kinase bind in the ATP site.* The Journal of biological chemistry, 1997. **272**(18): p. 12116-21.

54. Wilson, K.P., et al., *The structural basis for the specificity of pyridinylimidazole inhibitors of p38 MAP kinase.* Chemistry & biology, 1997. **4**(6): p. 423-31.

55. Roux, P.P. and J. Blenis, *ERK and p38 MAPK-activated protein kinases: a family of protein kinases with diverse biological functions.* Microbiology and molecular biology reviews : MMBR, 2004. **68**(2): p. 320-44.

56. Walsh, D. and I. Mohr, *Phosphorylation of eIF4E by Mnk-1 enhances HSV-1 translation and replication in quiescent cells.* Genes and Development, 2004. **18**: p. 660-672.

57. Gingras, A.C., et al., *Regulation of 4E-BP1 phosphorylation: a novel two-step mechanism.* Genes & development, 1999. **13**(11): p. 1422-37.

58. Hay, N. and N. Sonenberg, *Upstream and downstream of mTOR.* Genes & development, 2004. **18**(16): p. 1926-45.

59. Liu, J., et al., *Protein phosphatase PPM1G regulates protein translation and cell growth by dephosphorylating 4E binding protein 1 (4E-BP1).* The Journal of biological chemistry, 2013. **288**(32): p. 23225-33.

60. Brown, E.J., et al., *A mammalian protein targeted by G1-arresting rapamycin-receptor complex.* Nature, 1994. **369**(6483): p. 756-8.

61. Brown, E.J., et al., *Control of p70 s6 kinase by kinase activity of FRAP in vivo.* Nature, 1995. **377**(6548): p. 441-6.

62. Burnett, P.E., et al., *RAFT1 phosphorylation of the translational regulators p70 S6 kinase and 4E-BP1.* Proceedings of the National Academy of Sciences of the United States of America, 1998. **95**(4): p. 1432-7.

63. Byrd, M.P., M. Zamora, and R.E. Lloyd, *Generation of multiple isoforms of eukaryotic translation initiation factor 4GI by use of alternate translation initiation codons.* Molecular and cellular biology, 2002. **22**(13): p. 4499-511.

64. Raught, B., et al., *Serum-stimulated, rapamycin-sensitive phosphorylation sites in the eukaryotic translation initiation factor 4GI.* The EMBO journal, 2000. **19**(3): p. 434-44.

65. Honess, R.W. and B. Roizman, *Regulation of herpesvirus macromolecular synthesis. I. Cascade regulation of the synthesis of three groups of viral proteins.* Journal of virology, 1974. **14**(1): p. 8-19.

66. Sze, P. and R.C. Herman, *The herpes simplex virus type 1 ICP6 gene is regulated by a 'leaky' early promoter.* Virus research, 1992. **26**(2): p. 141-52.

67. Fields, B.N., *Fields Virology Volume 2*. 3rd ed. Fields Virology. Vol. 2. 1996, Philadelphia: Lippincott-Raven Publishers. 8.

68. Walsh, D. and I. Mohr, *Assembly of an active translation initiation factor complex by a viral protein.* Genes & development, 2006. **20**(4): p. 461-72.

69. Cameron, J.M., et al., *Ribonucleotide reductase encoded by herpes simplex virus is a determinant of the pathogenicity of the virus in mice and a valid antiviral target.* The Journal of general virology, 1988. **69 (Pt 10)**: p. 2607-12.

70. Jacobson, J.G., et al., *A herpes simplex virus ribonucleotide reductase deletion mutant is defective for productive acute and reactivatable latent infections of mice and for replication in mouse cells.* Virology, 1989. **173**(1): p. 276-83.

71. Purves, F.C., D. Spector, and B. Roizman, *The herpes simplex virus 1 protein kinase encoded by the US3 gene mediates posttranslational modification of the phosphoprotein encoded by the UL34 gene.* Journal of virology, 1991. **65**(11): p. 5757-64.

72. Purves, F.C., et al., *Herpes simplex virus 1 protein kinase is encoded by open reading frame US3 which is not essential for virus growth in cell culture.* Journal of virology, 1987. **61**(9): p. 2896-901.

73. Frame, M.C., et al., *Identification of the herpes simplex virus protein kinase as the product of viral gene US3.* The Journal of general virology, 1987. **68 (Pt 10)**: p. 2699-704.

74. Reynolds, A.E., et al., *U(L)31 and U(L)34 proteins of herpes simplex virus type 1 form a complex that accumulates at the nuclear rim and is required for envelopment of nucleocapsids.* Journal of virology, 2001. **75**(18): p. 8803-17.

75. Chuluunbaatar, U. and I. Mohr, *A herpesvirus kinase that masquerades as Akt: you don't have to look like Akt, to act like it.* Cell cycle, 2011. **10**(13): p. 2064-8.

76. Eaton, H.E., et al., *Herpes simplex virus protein kinases US3 and UL13 modulate VP11/12 phosphorylation, virion packaging, and phosphatidylinositol 3-kinase/Akt signaling activity.* Journal of virology, 2014. **88**(13): p. 7379-88.

77. Cully, M., et al., *A role for p38 stress-activated protein kinase in regulation of cell growth via TORC1.* Molecular and cellular biology, 2010. **30**(2): p. 481-95.

78. *Lipofectamine Reagent*. 2004 09 July 2004 [cited 2011 October 20]; Available from: tools.invitrogen.com/content/sfs/manuals/lipofectamine_man.pdf.

79. Qin, H., et al., *Phosphorylation screening identifies translational initiation factor 4GII as an intracellular target of Ca(2+)/calmodulin-dependent protein kinase I.* The Journal of biological chemistry, 2003. **278**(49): p. 48570-9.

80. Marston, H.D., et al., *Emerging viral diseases: confronting threats with new technologies.* Sci Transl Med, 2014. **6**(253): p. 253ps10.

81. Jones, K.E., et al., *Global trends in emerging infectious diseases.* Nature, 2008. **451**(7181): p. 990-3.

82. Taylor, L.H., S.M. Latham, and M.E. Woolhouse, *Risk factors for human disease emergence.* Philos Trans R Soc Lond B Biol Sci, 2001. **356**(1411): p. 983-9.

83. Luby, S.P., et al., *Foodborne transmission of Nipah virus, Bangladesh.* Emerg Infect Dis, 2006. **12**(12): p. 1888-94.

84. Biek, R., et al., *Recent common ancestry of Ebola Zaire virus found in a bat reservoir.* PLoS Pathog, 2006. **2**(10): p. e90.

85. Pigott, D.M., et al., *Mapping the zoonotic niche of Ebola virus disease in Africa.* Elife, 2014. **3**: p. e04395.

86. Antia, R., et al., *The role of evolution in the emergence of infectious diseases.* Nature, 2003. **426**(6967): p. 658-61.

87. Holmes, E.C. and A.J. Drummond, *The evolutionary genetics of viral emergence.* Curr Top Microbiol Immunol, 2007. **315**: p. 51-66.

88. Chen, Z., et al., *MAP kinases.* Chem Rev, 2001. **101**(8): p. 2449-76.

89. Kyriakis, J.M. and J. Avruch, *Mammalian mitogen-activated protein kinase signal transduction pathways activated by stress and inflammation.* Physiol Rev, 2001. **81**(2): p. 807-69.

90. Pearson, G., et al., *Mitogen-activated protein (MAP) kinase pathways: regulation and physiological functions.* Endocr Rev, 2001. **22**(2): p. 153-83.

91. Beardmore, V.A., et al., *Generation and characterization of p38beta (MAPK11) gene-targeted mice.* Mol Cell Biol, 2005. **25**(23): p. 10454-64.

92. Choy, E.H. and G.S. Panayi, *Cytokine pathways and joint inflammation in rheumatoid arthritis.* N Engl J Med, 2001. **344**(12): p. 907-16.

93. Gardiner, T.A., et al., *Inhibition of tumor necrosis factor-alpha improves physiological angiogenesis and reduces pathological neovascularization in ischemic retinopathy.* Am J Pathol, 2005. **166**(2): p. 637-44.

94. Lee, M.R. and C. Dominguez, *MAP kinase p38 inhibitors: clinical results and an intimate look at their interactions with p38alpha protein.* Curr Med Chem, 2005. **12**(25): p. 2979-94.

95. Borgeling, Y., et al., *Inhibition of p38 mitogen-activated protein kinase impairs influenza virus-induced primary and secondary host gene responses and protects mice from lethal H5N1 infection.* J Biol Chem, 2014. **289**(1): p. 13-27.

96. Raught, B. and A.C. Gingras, *eIF4E activity is regulated at multiple levels.* Int J Biochem Cell Biol, 1999. **31**(1): p. 43-57.

97. Frederickson, R.M., W.E. Mushynski, and N. Sonenberg, *Phosphorylation of translation initiation factor eIF-4E is induced in a ras-dependent manner during nerve growth factor-mediated PC12 cell differentiation.* Mol Cell Biol, 1992. **12**(3): p. 1239-47.

98. Diaz-Meco, M.T., et al., *Evidence for the in vitro and in vivo interaction of Ras with protein kinase C zeta.* J Biol Chem, 1994. **269**(50): p. 31706-10.